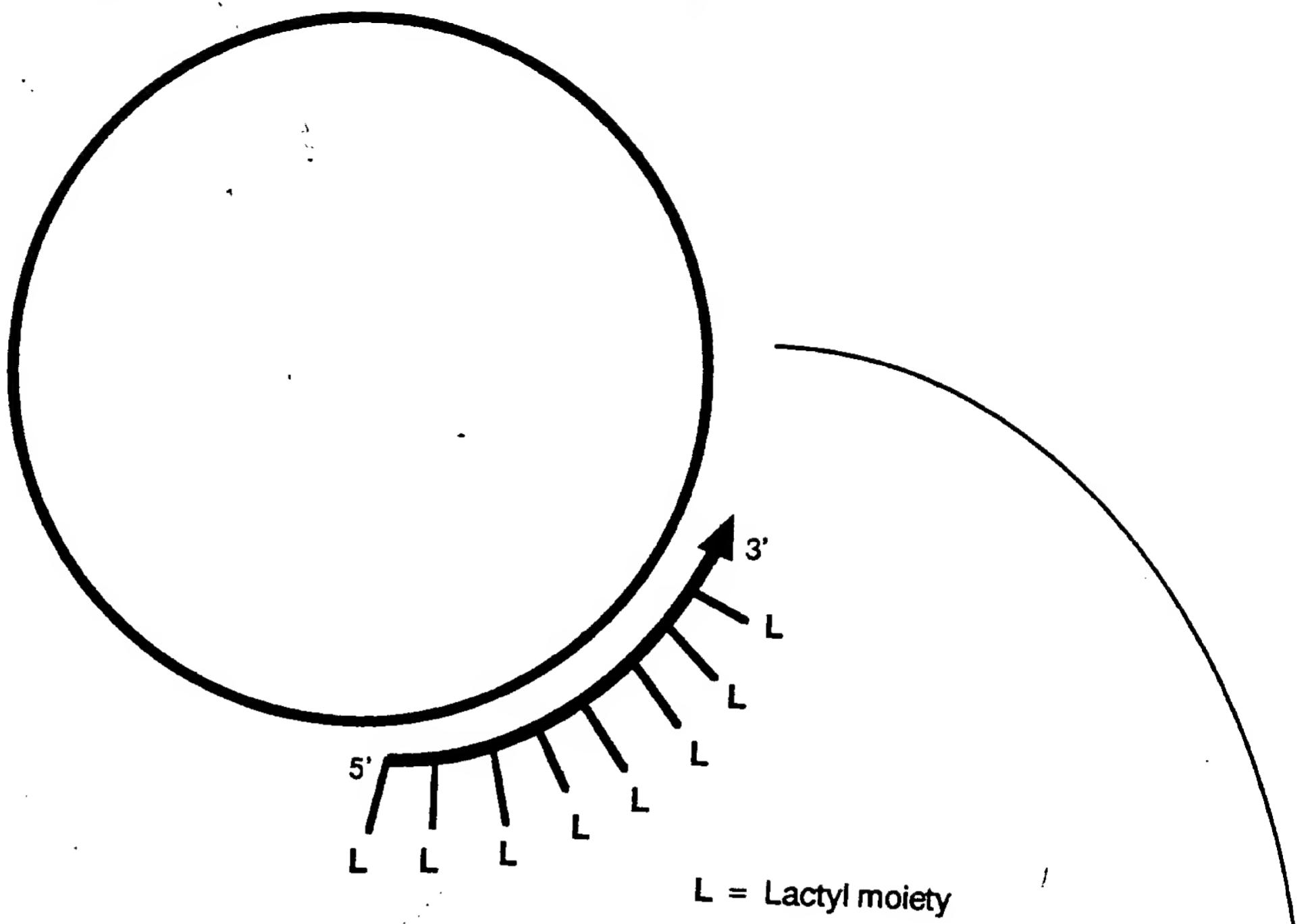


(a)



(b)

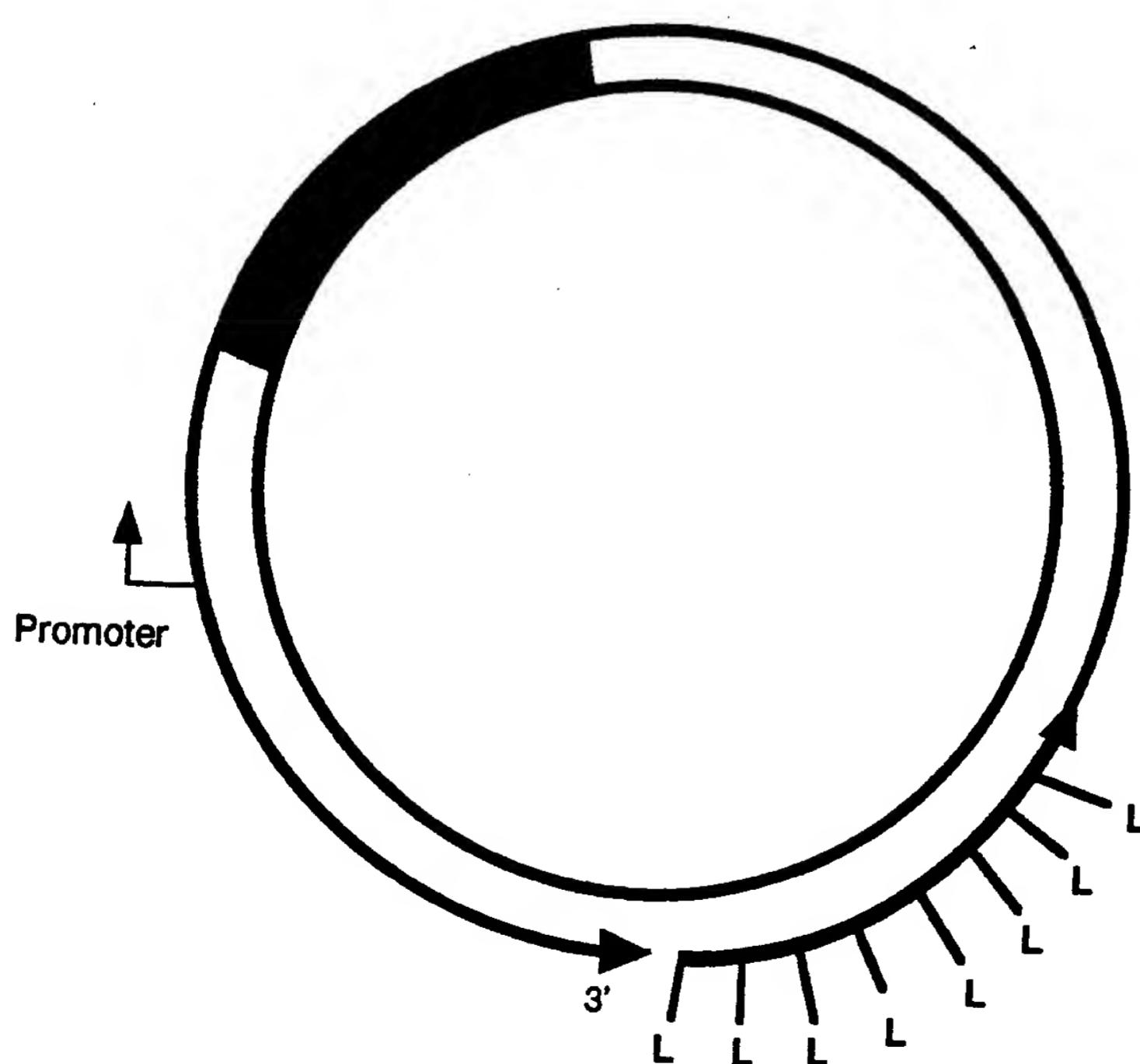


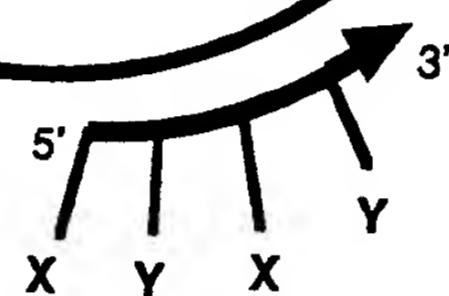
Figure I

Attachment of Ligands Through Primer Region

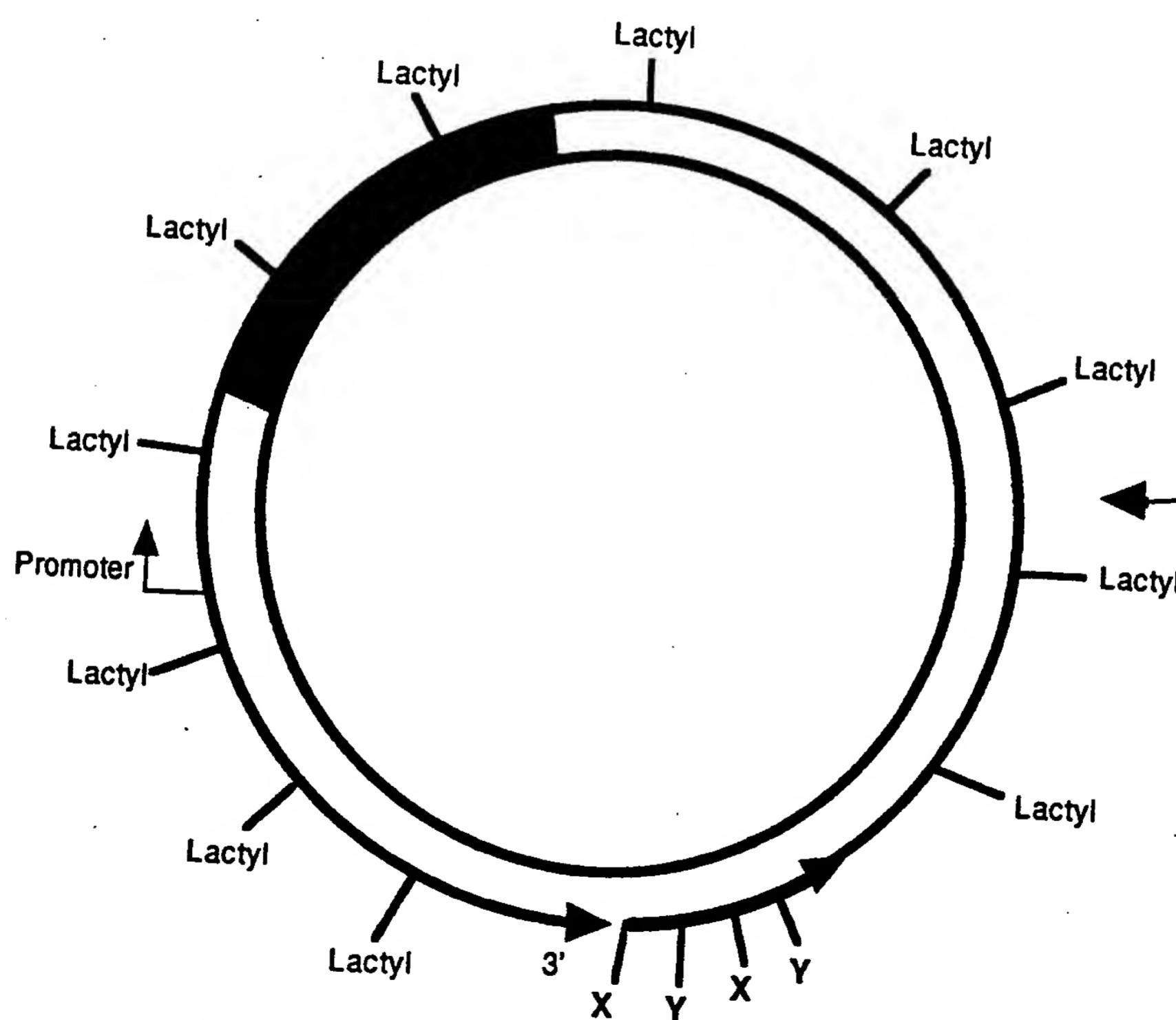
(a)

X = Nuclear Localisation Signal

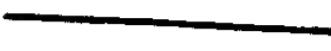
Y = fusogenic peptide



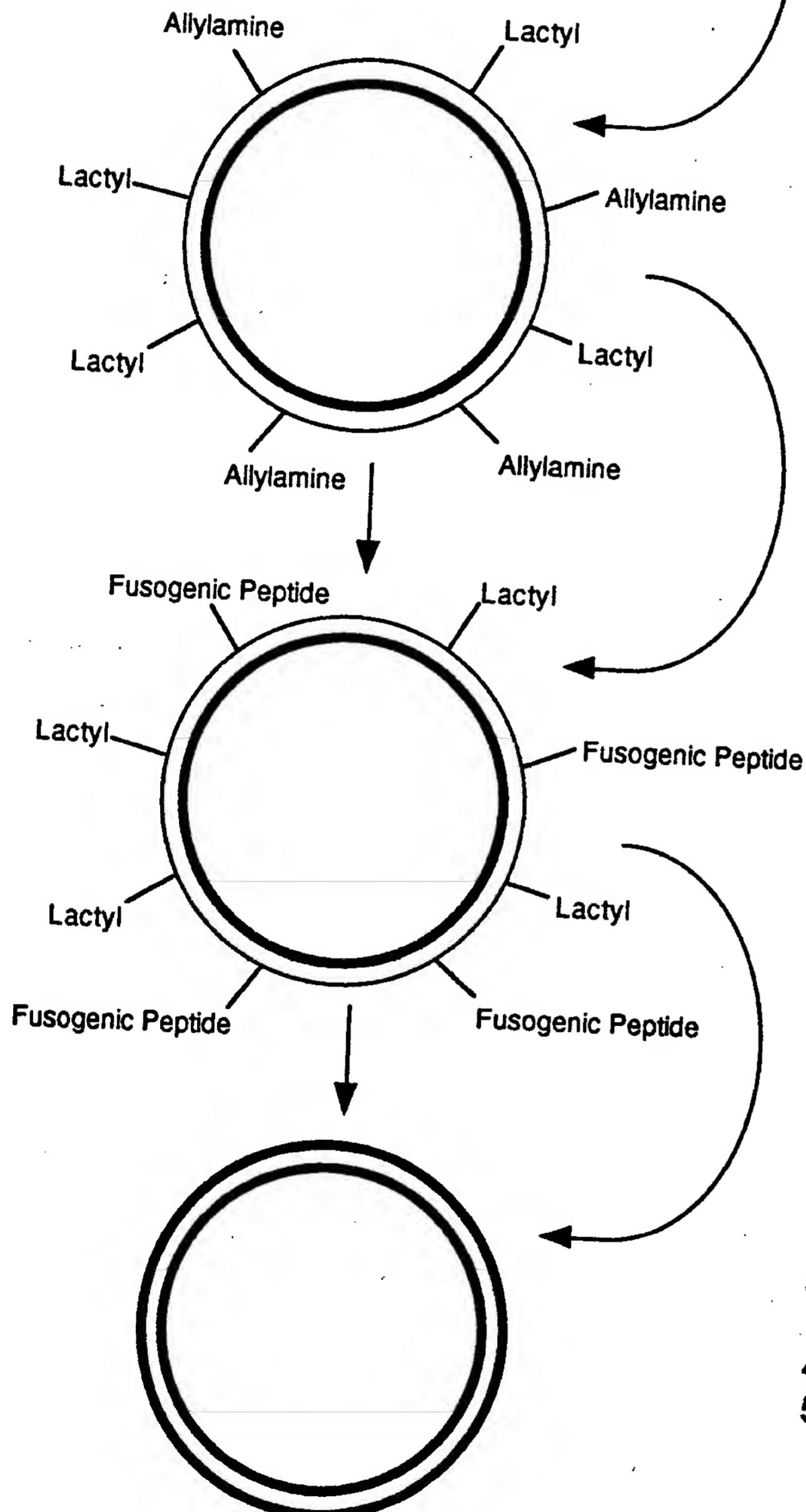
(b)



**Figure 2**  
Attachment of Ligands by Incorporation of Modified Nucleotide Precursors

 = DNA  
 = RNA

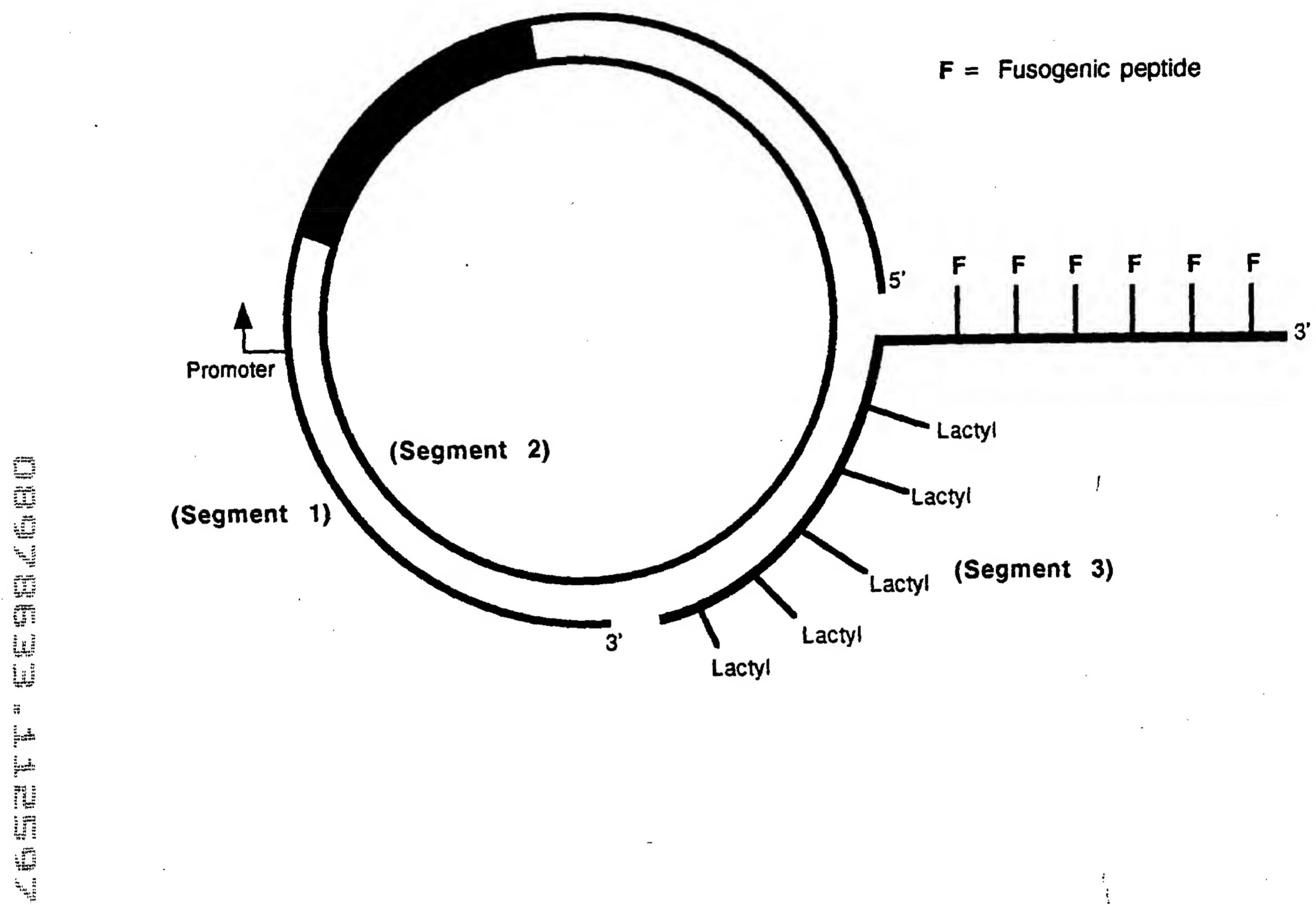
Synthesis of RNA using lactyl-UTP and Allylamine-UTP precursors



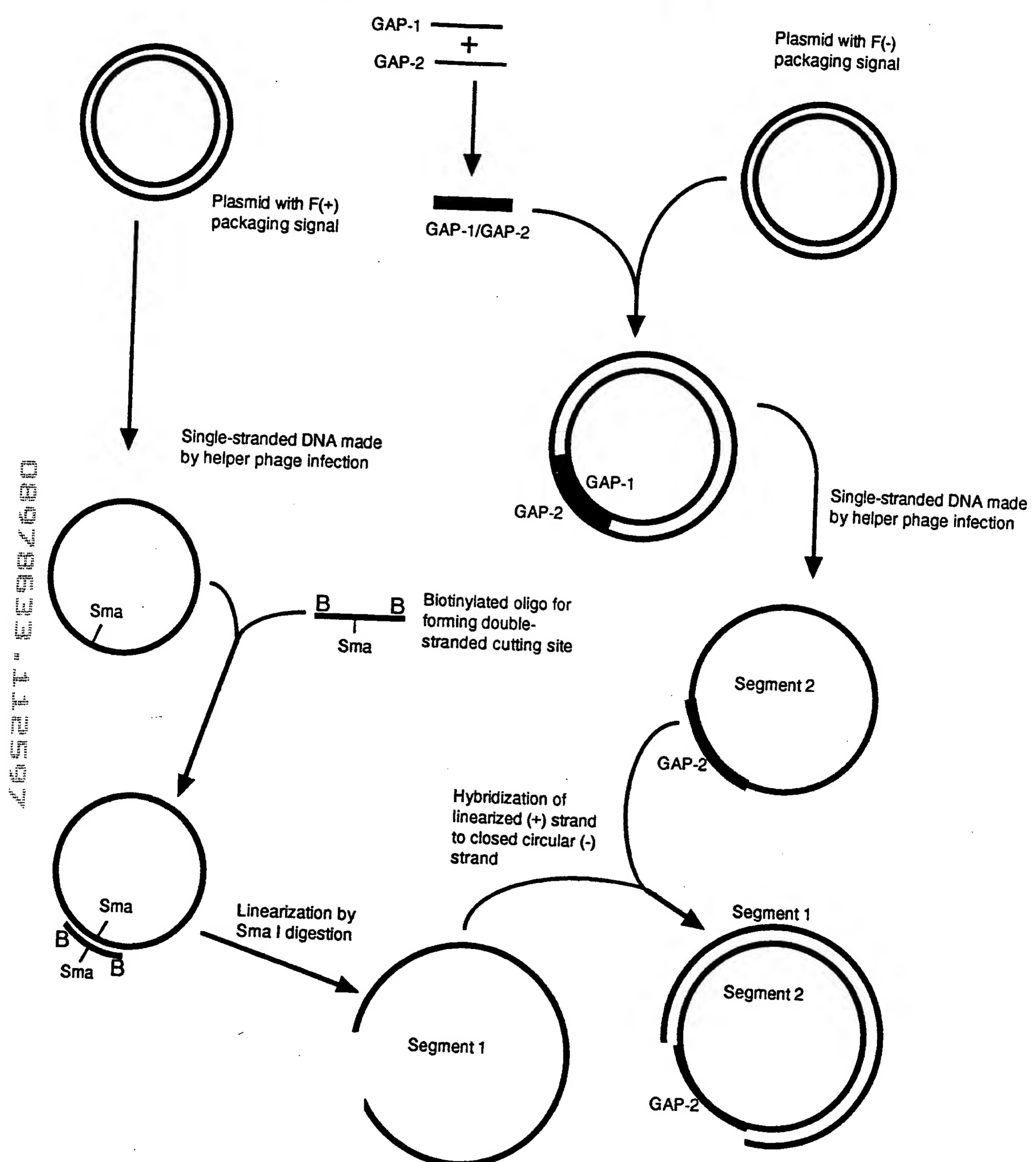
Attachment of fusogenic peptide through allylamine linkage

- 1) attachment of construct to cell surface
- 2) endocytosis of construct
- 3) release of construct from endosome by means of fusogenic peptide
- 4) Elimination of RNA moieties by RNaseH
- 5) Synthesis of complimentary DNA strand

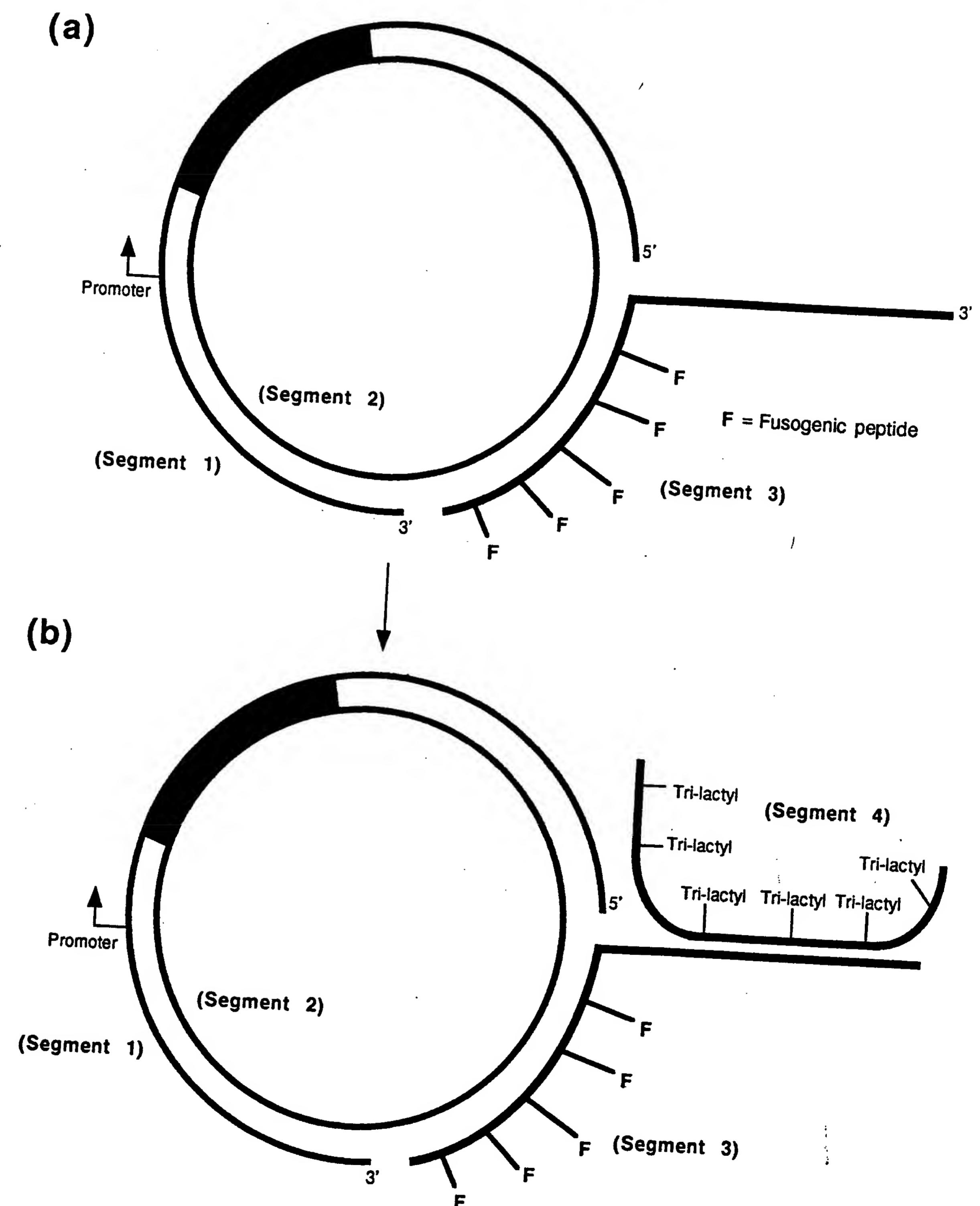
**Figure 3**  
Incorporation of Ligands through Modified Ribonucleotides



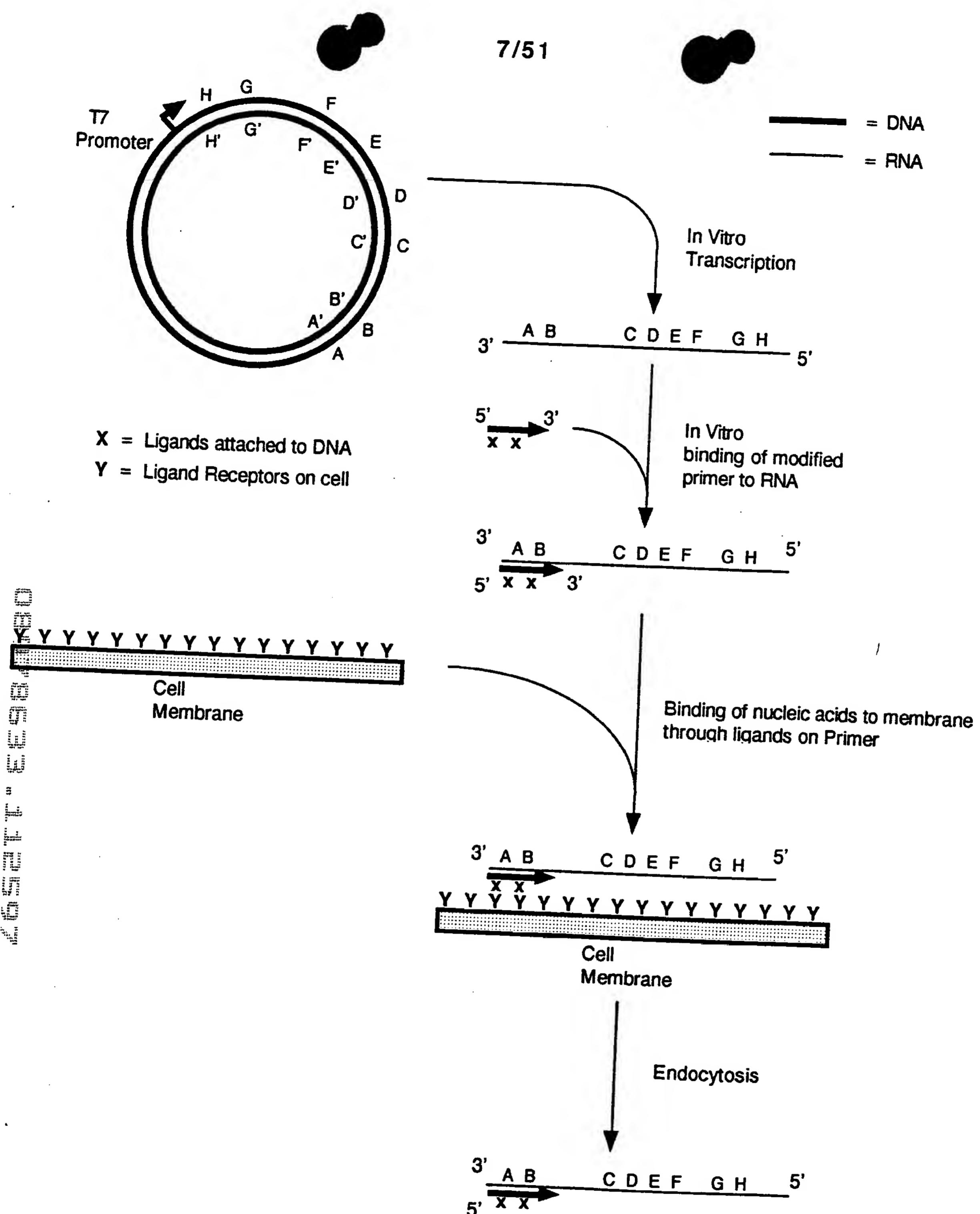
**Figure 4**  
Attachment of Ligands through a 3' tail



**Figure 5**  
Preparation of Gapped Circle



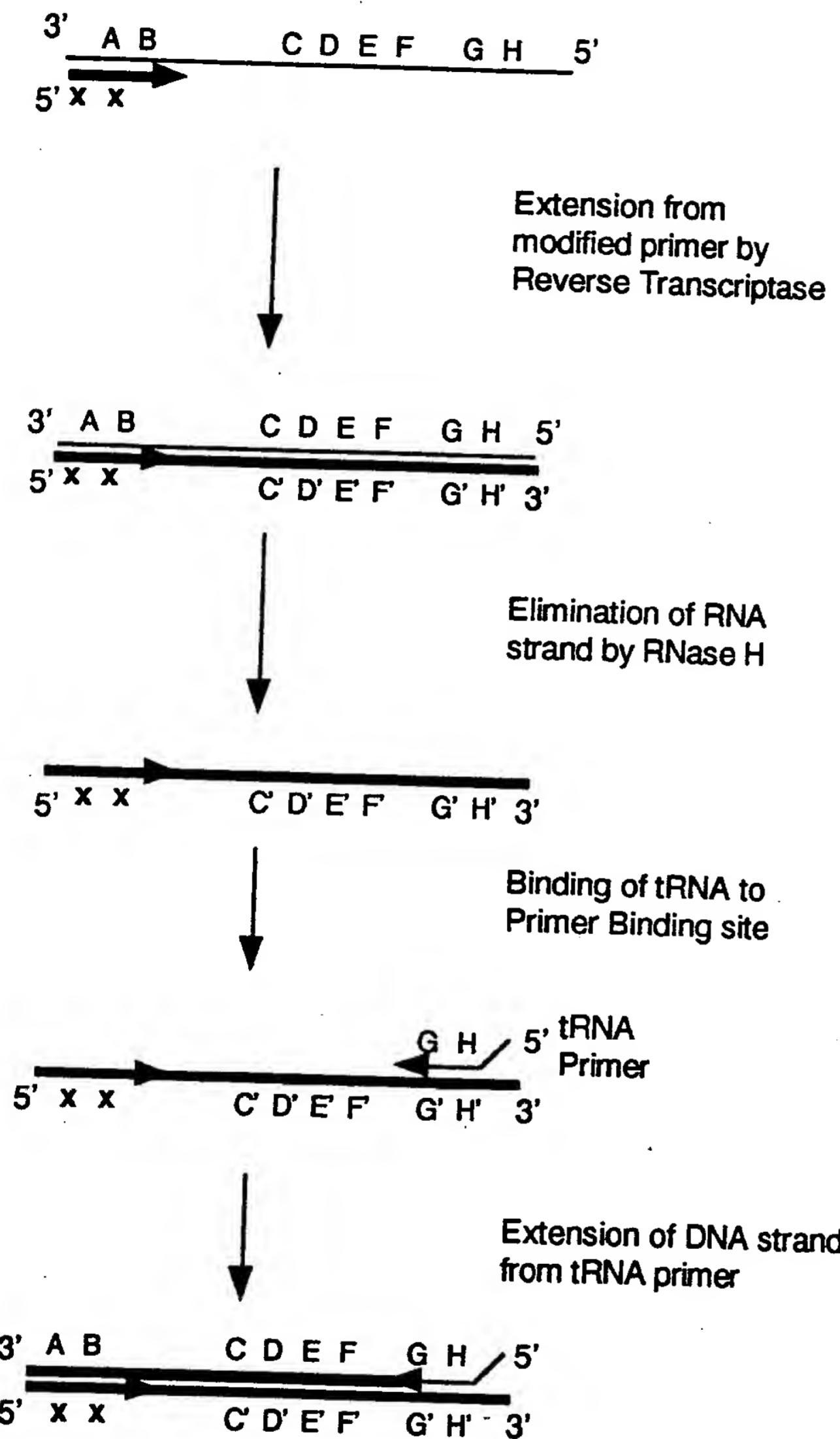
**Figure 6**  
Attachment of Ligands through hybridization to a 3' tail



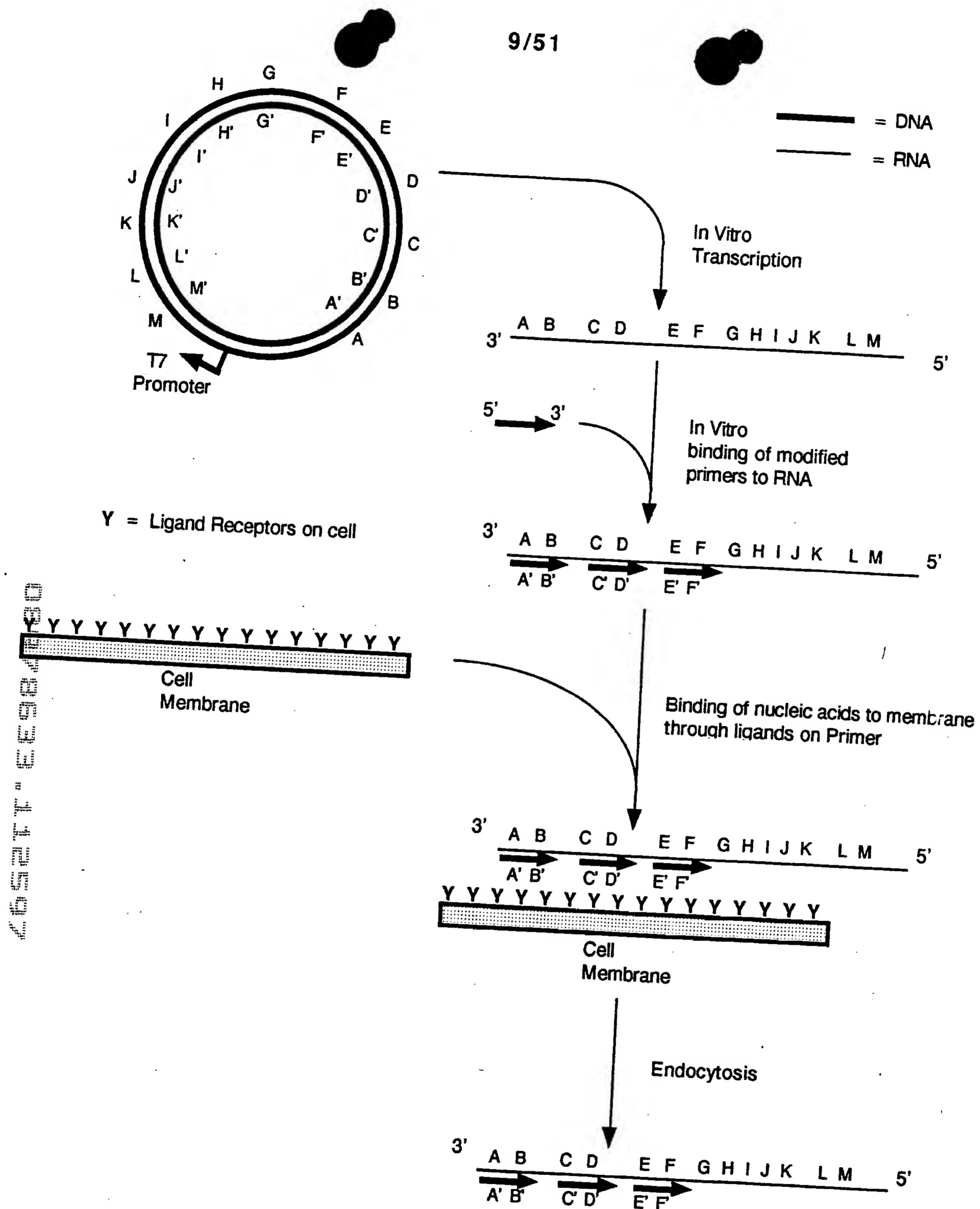
**Figure 7**  
**RNA with Ligands on Primer**

(Continued in Figure 8)

Continued from Figure 7



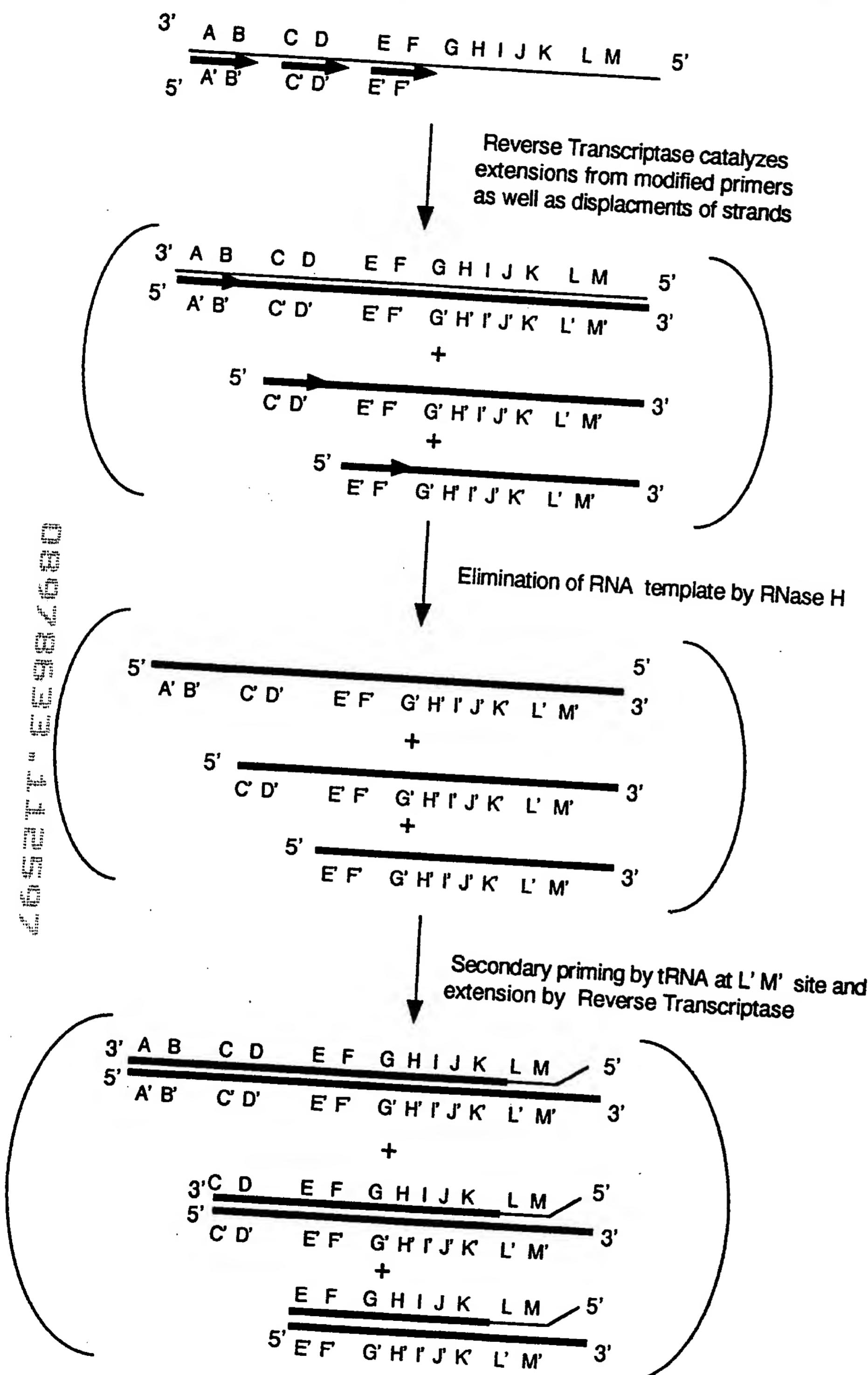
**Figure 8**  
RNA with Ligands on Primer (Continued)



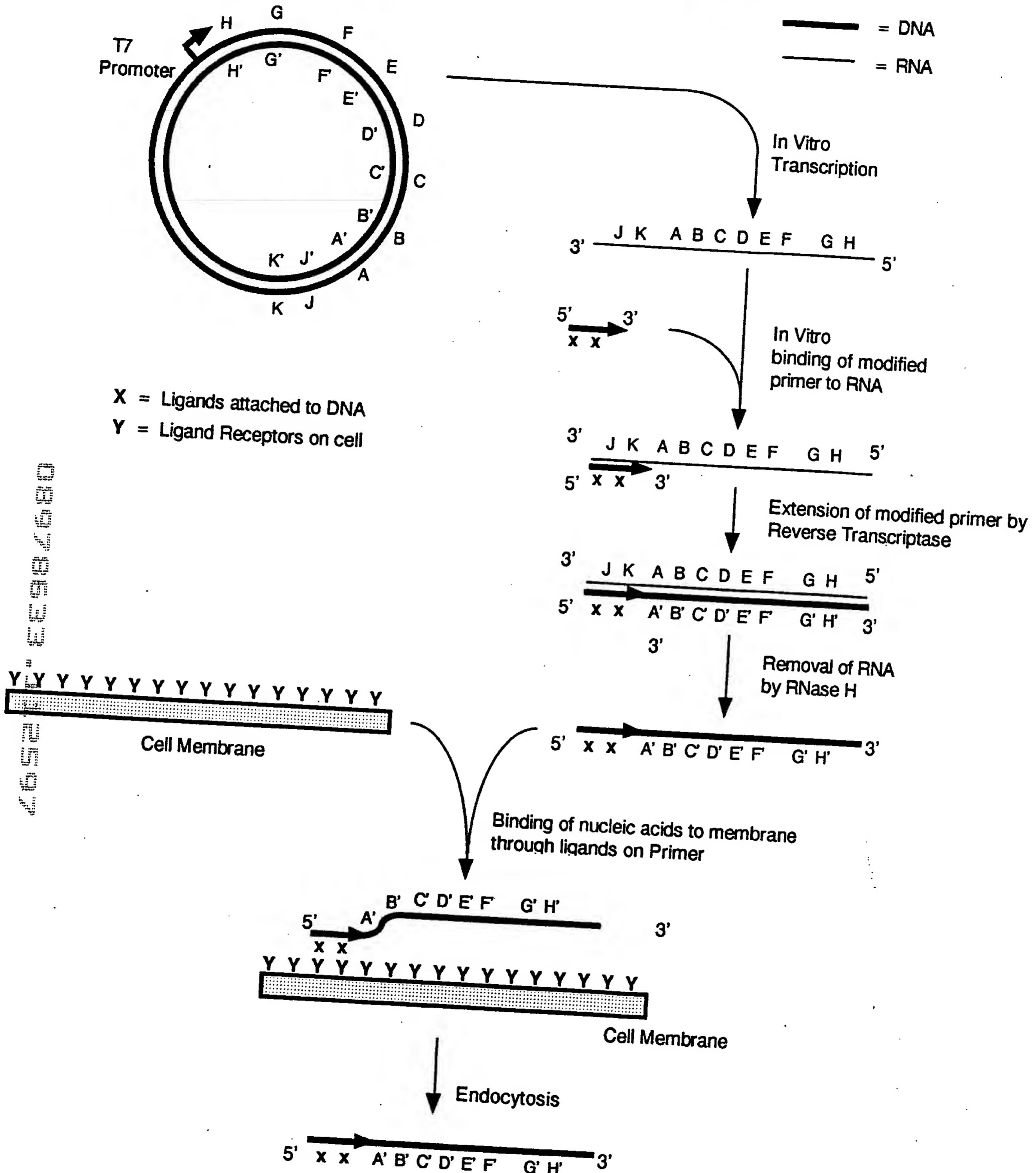
(Continued in Figure 10)

**Figure 9**  
RNA with Ligands on Multiple Primers

Continued from Figure 9



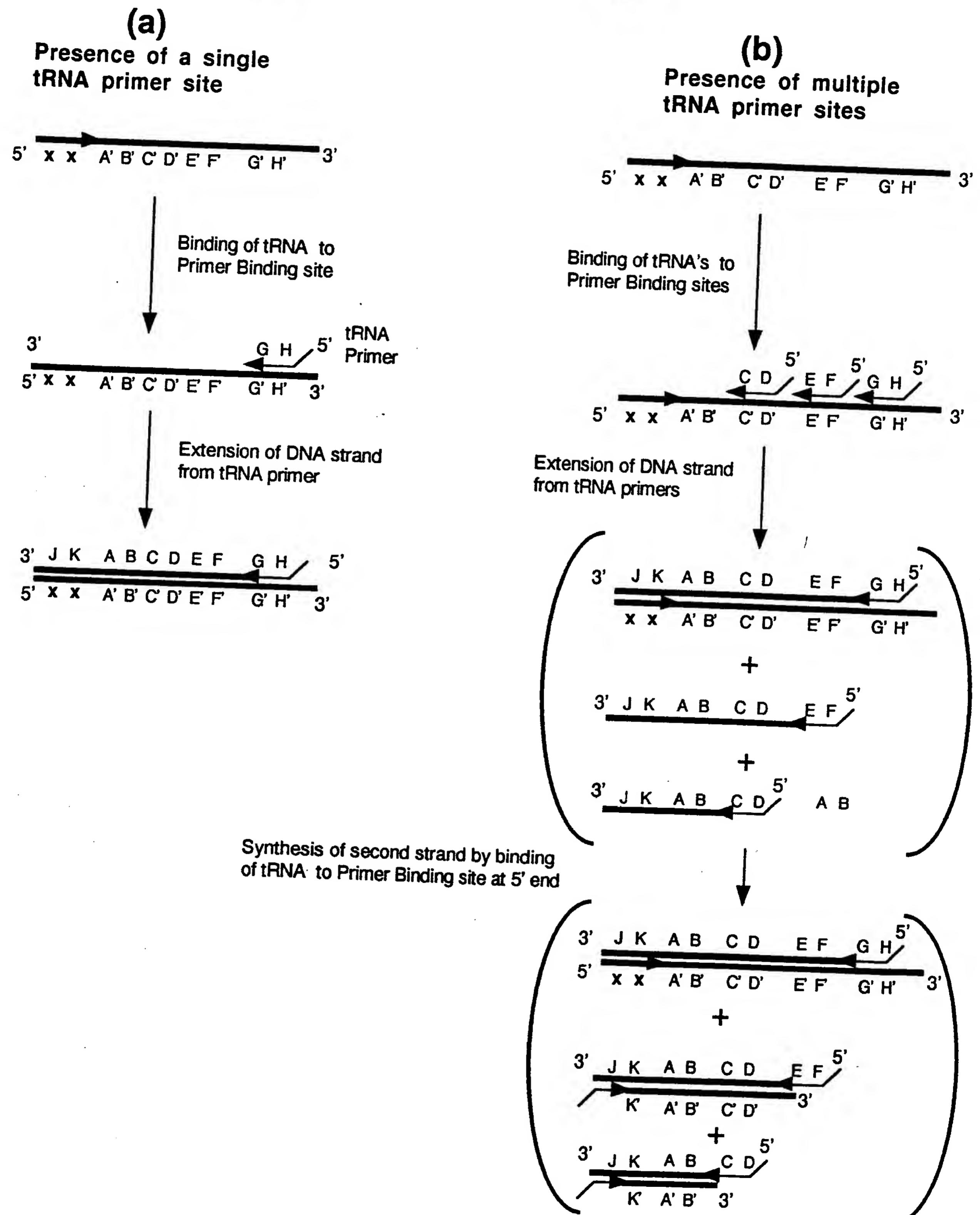
**Figure 10**  
RNA with Ligands on Multiple Primers (Continued)



(Continued in Figure 12)

**Figure 11**  
**Single-stranded DNA with attached Ligands**

Continued from Figure 11

**Figure 12****Single-stranded DNA with attached Ligands (continued)**

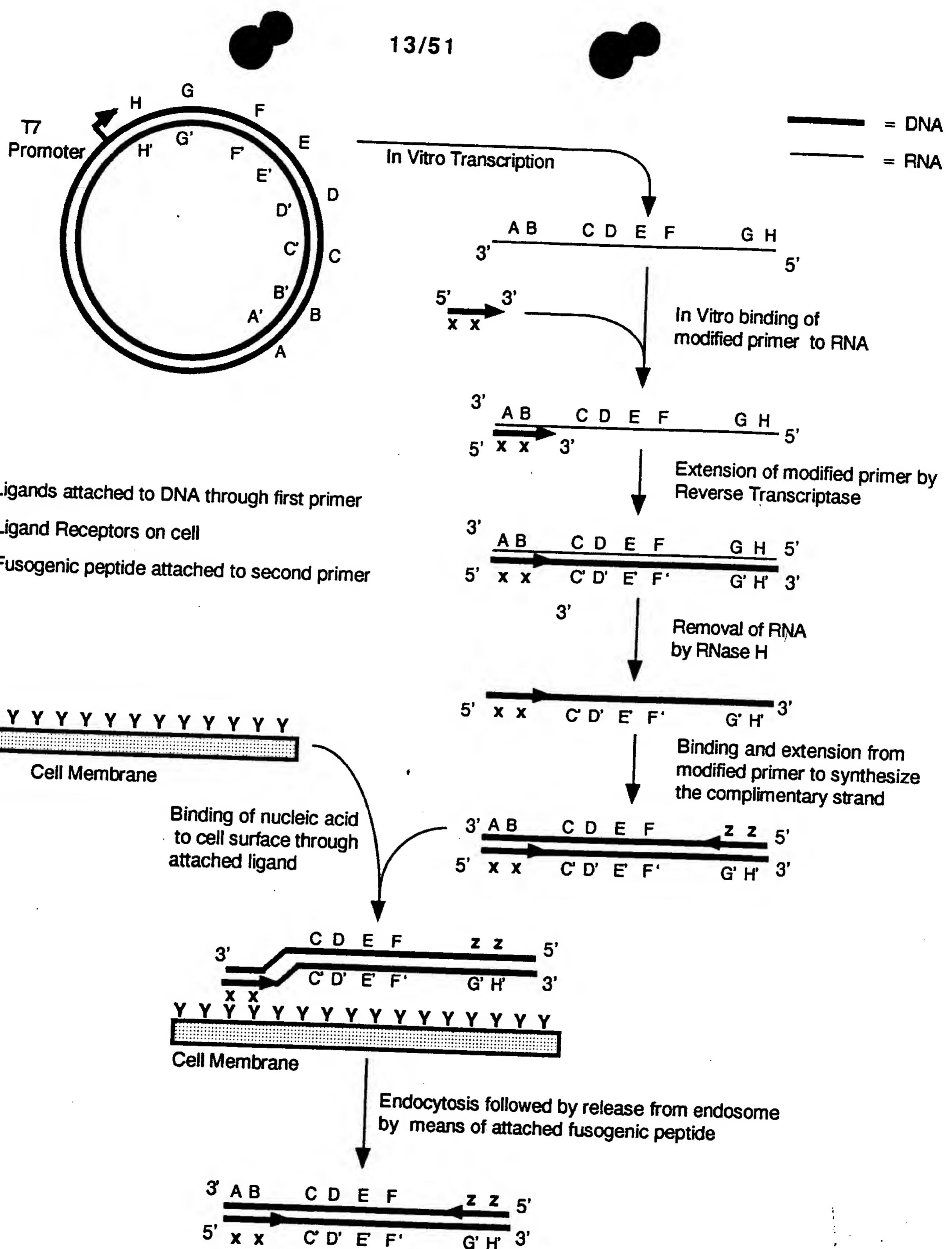
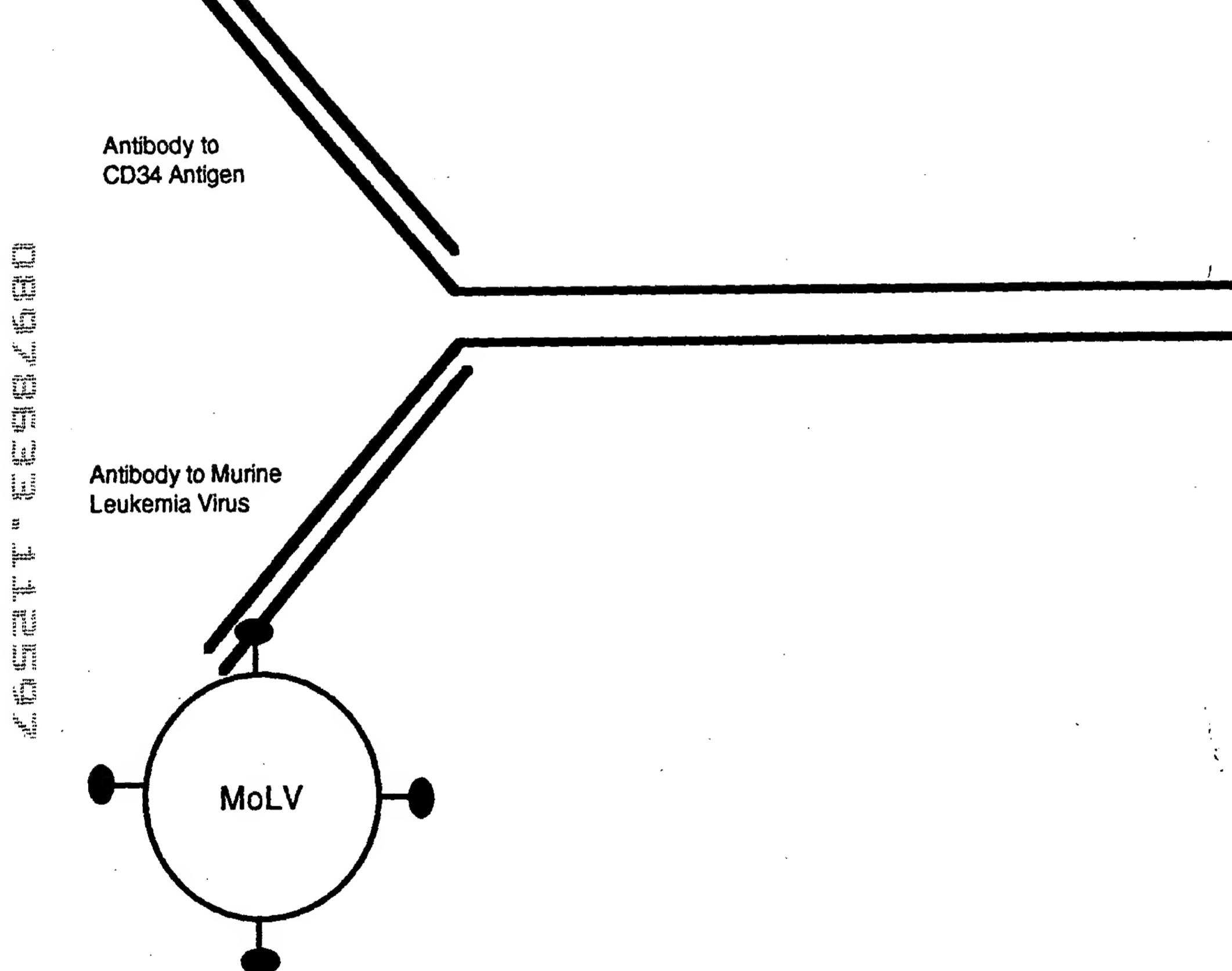


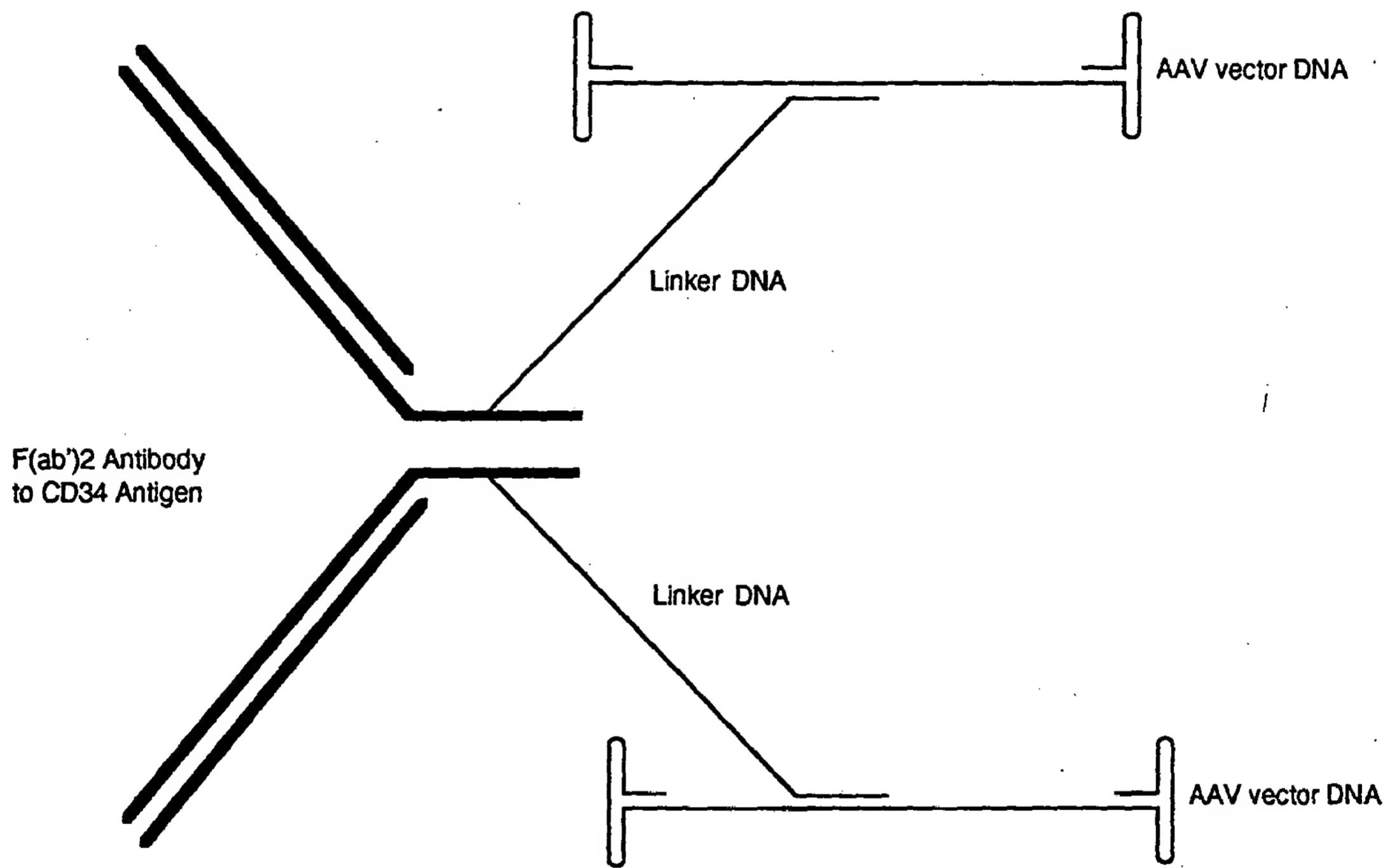
Figure 13

Linear Double-stranded DNA with attached Moieties on each strand



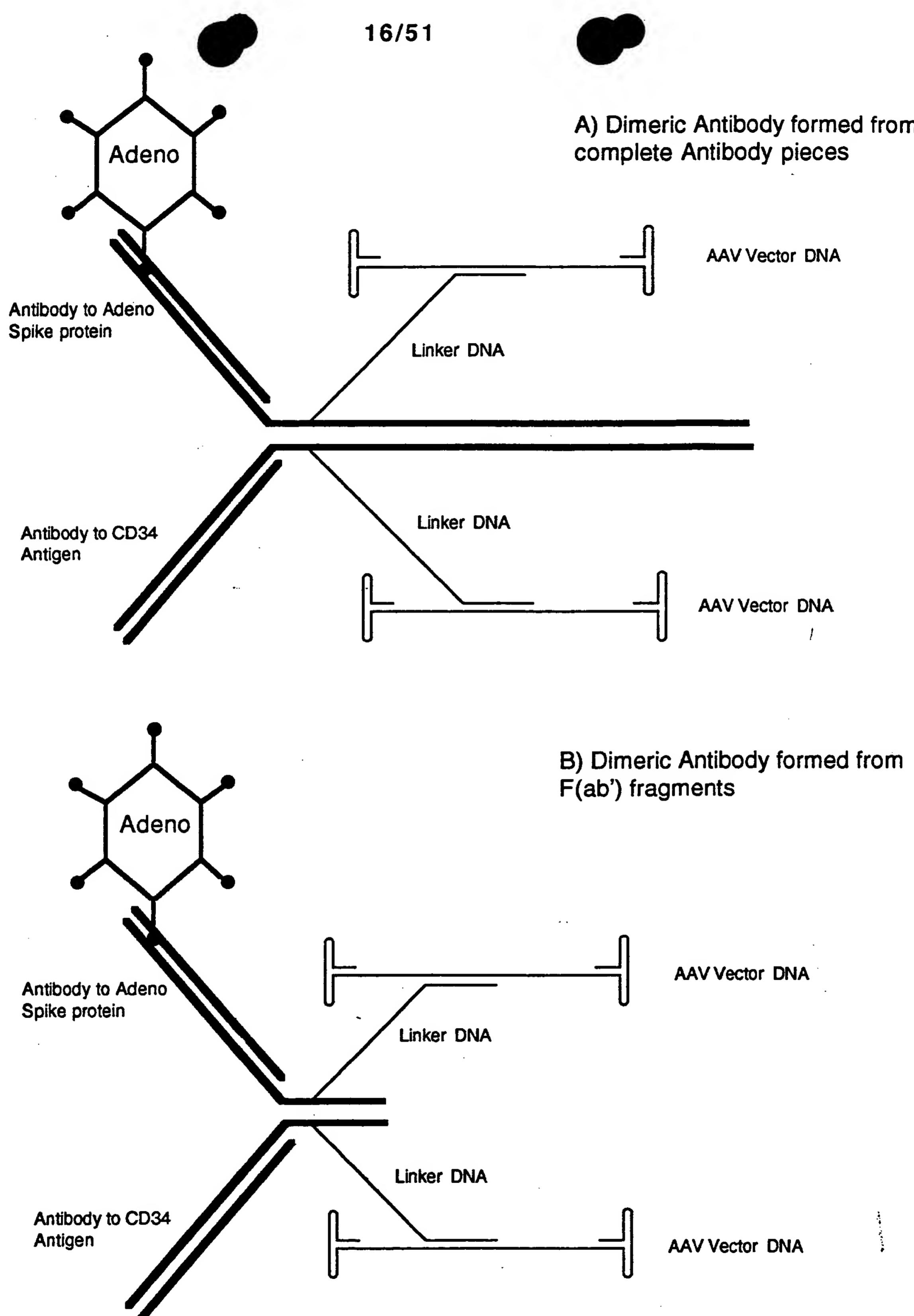
**Figure 14**  
Enhanced Delivery of Retroviral Vector  
to Haematopoietic Stem Cell

© 1992 Wiley-Liss, Inc.

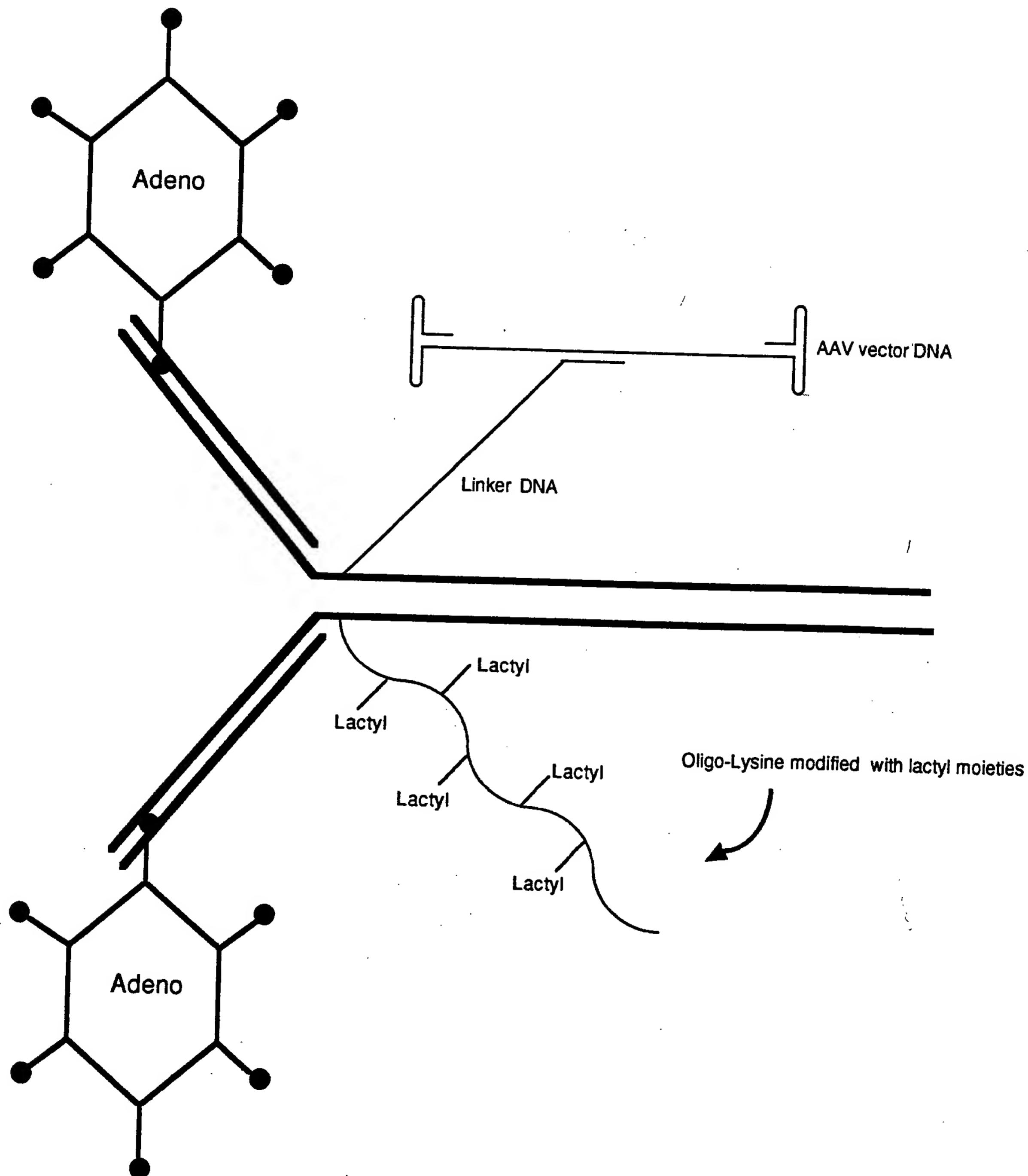


**Figure 15**

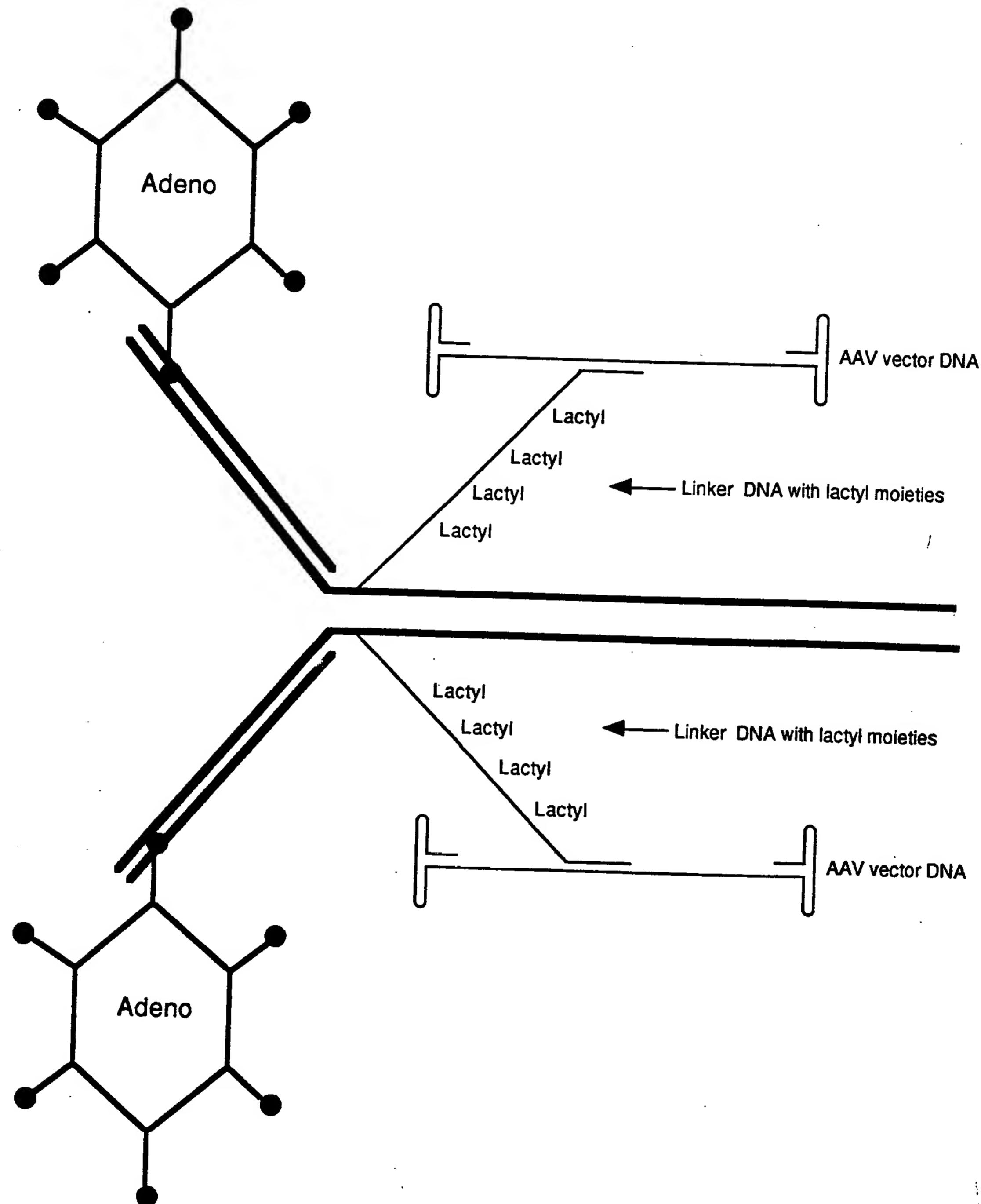
**Enhanced Delivery of Vector  
DNA to Haematopoietic Stem Cell**



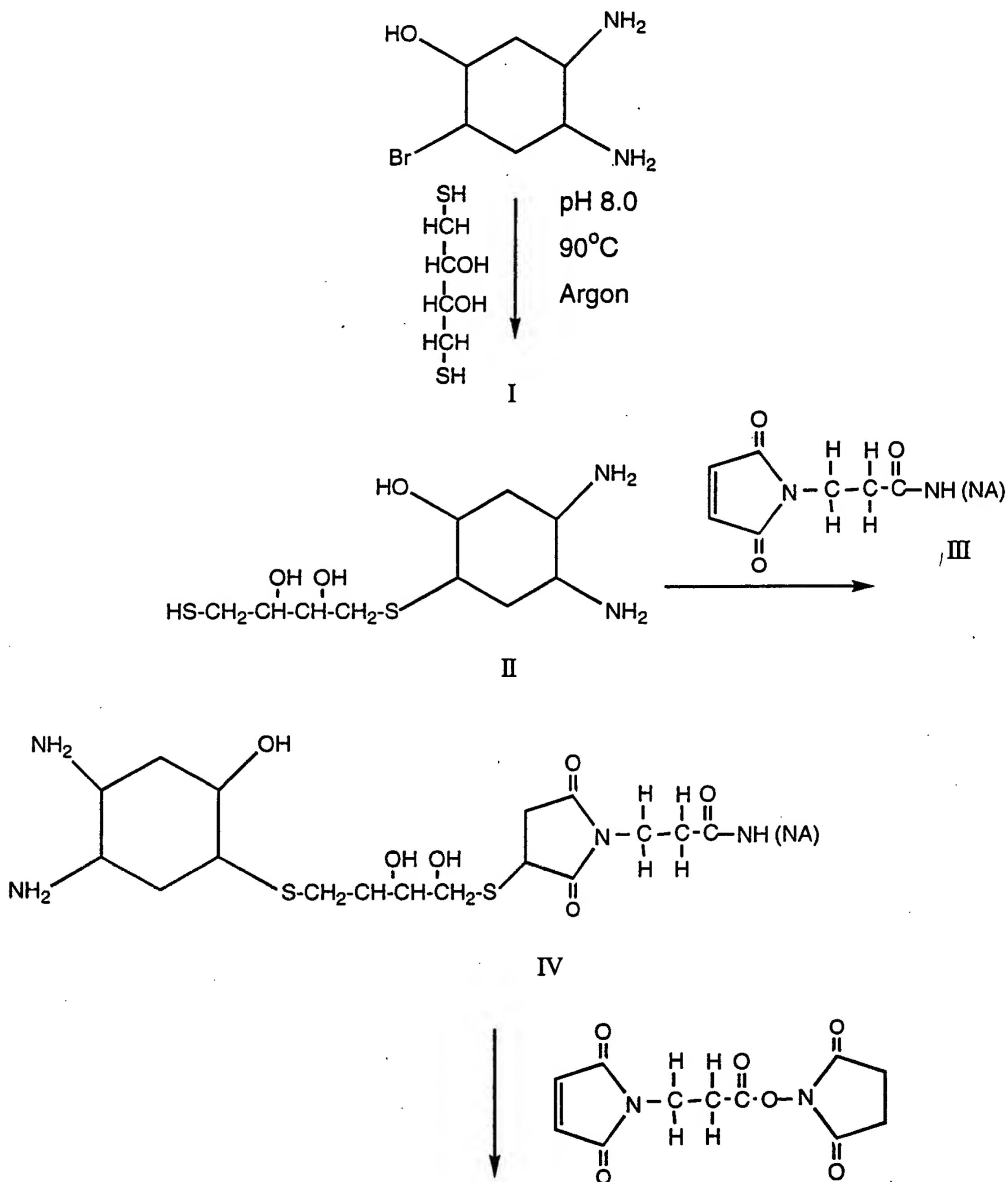
**Figure 16**  
Covalent Attachment of vector DNA to Dimeric Antibody



**Figure 17**  
Covalent attachment of Modified DNA  
to a Monovalent Antibody



**Figure 18**  
Modified DNA used as a Binder

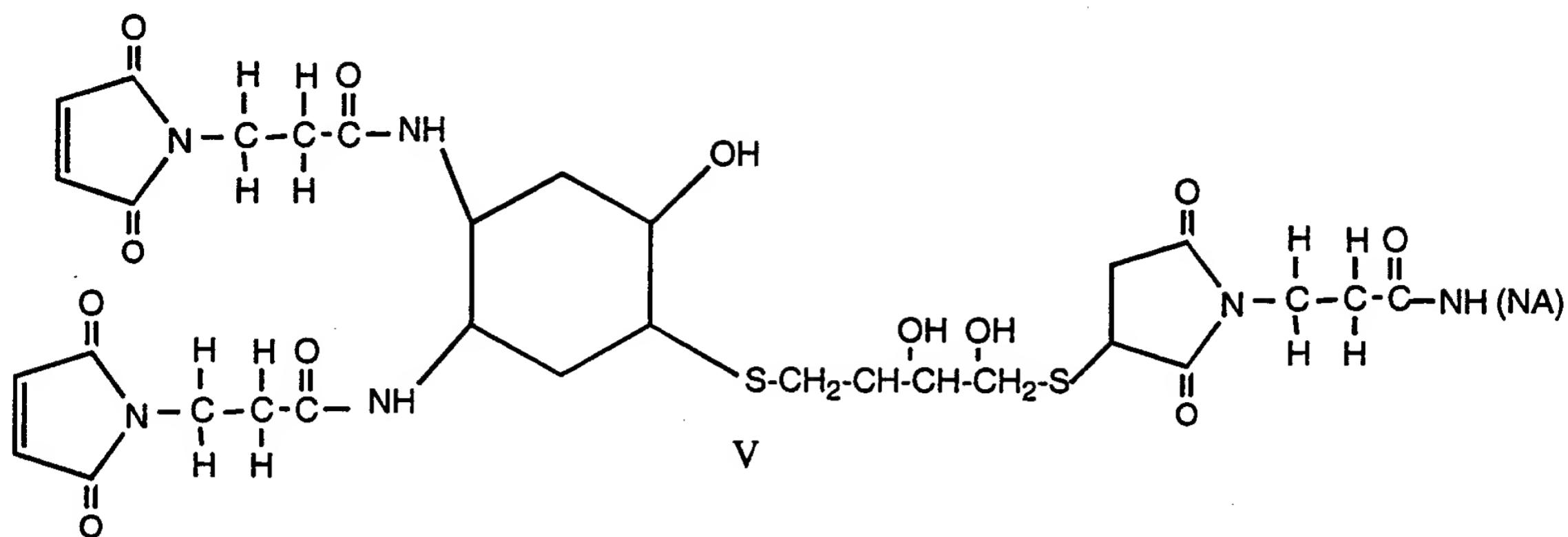


(continued in Figure 20)

**Figur 19**  
**Synthetic Steps for Creation of Antibodies**  
**With Nucleic Acid Moieties Attached**

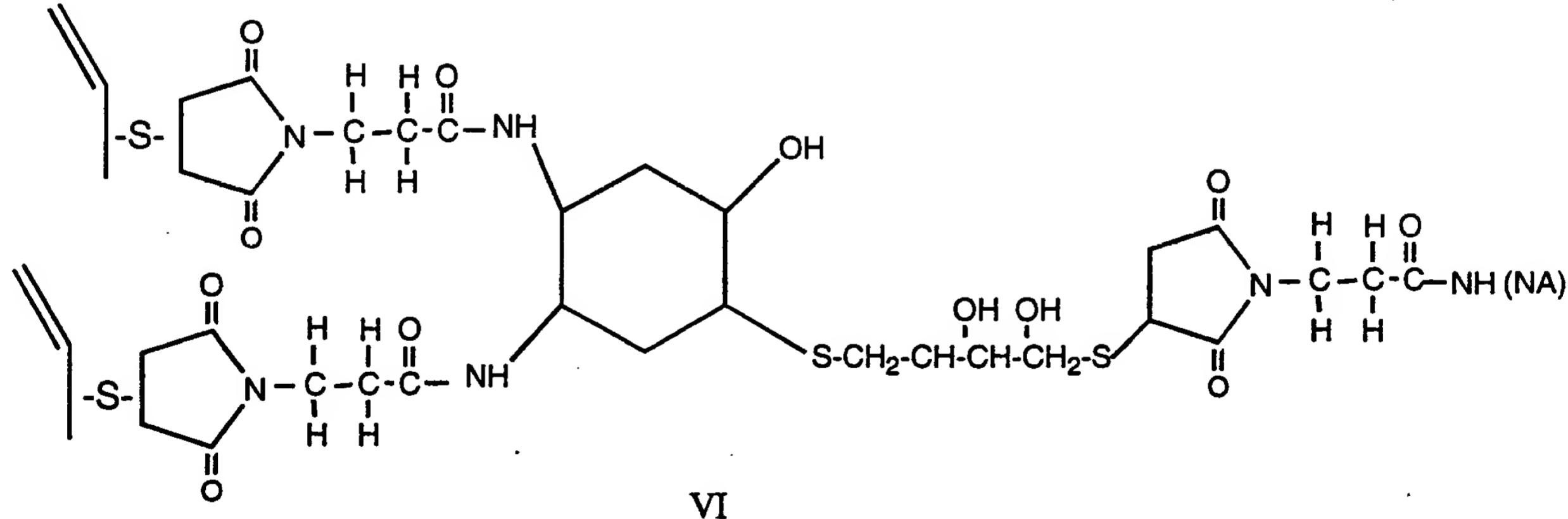
(Continued from Figure 19)

20/51



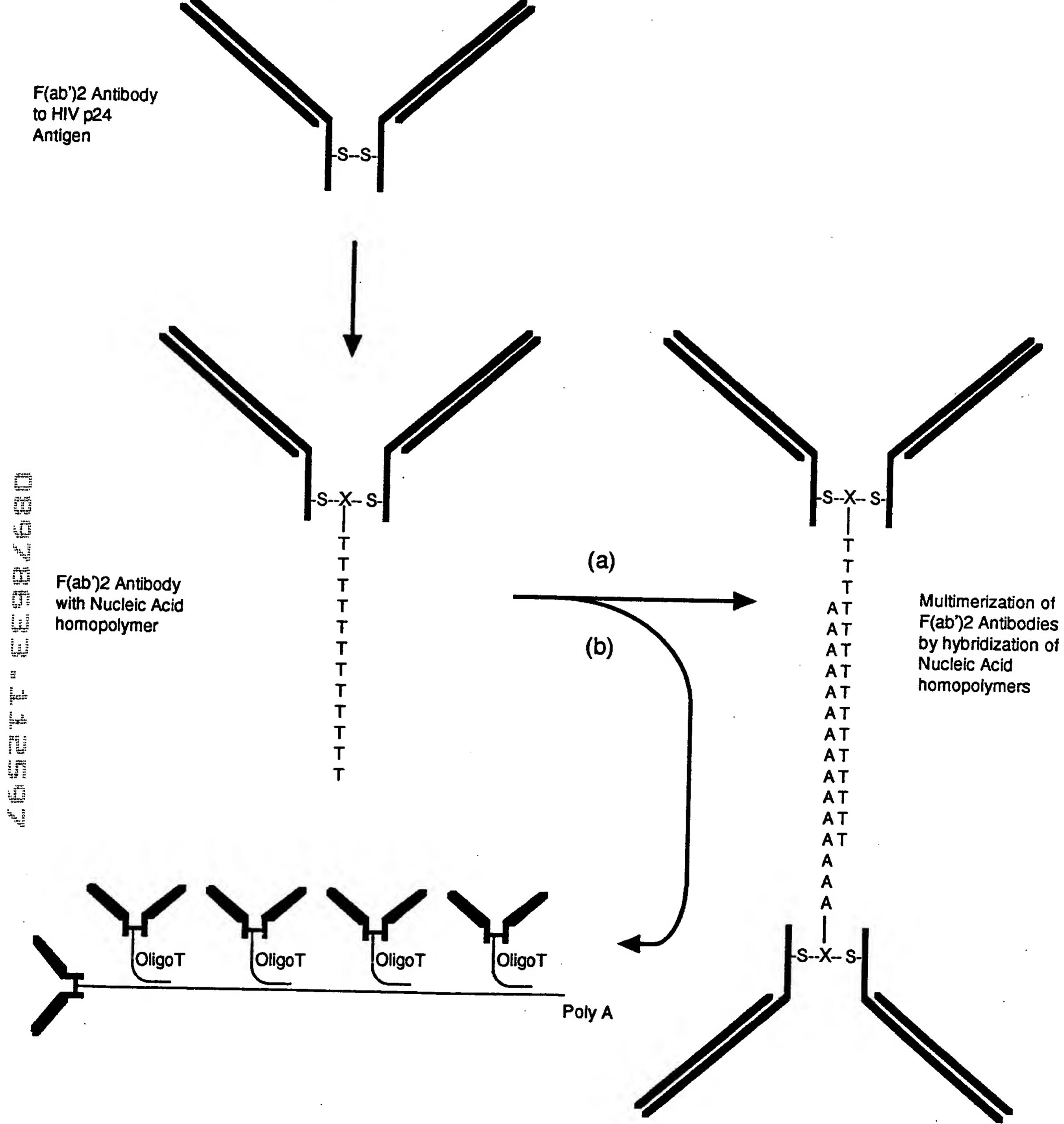
V

-SH



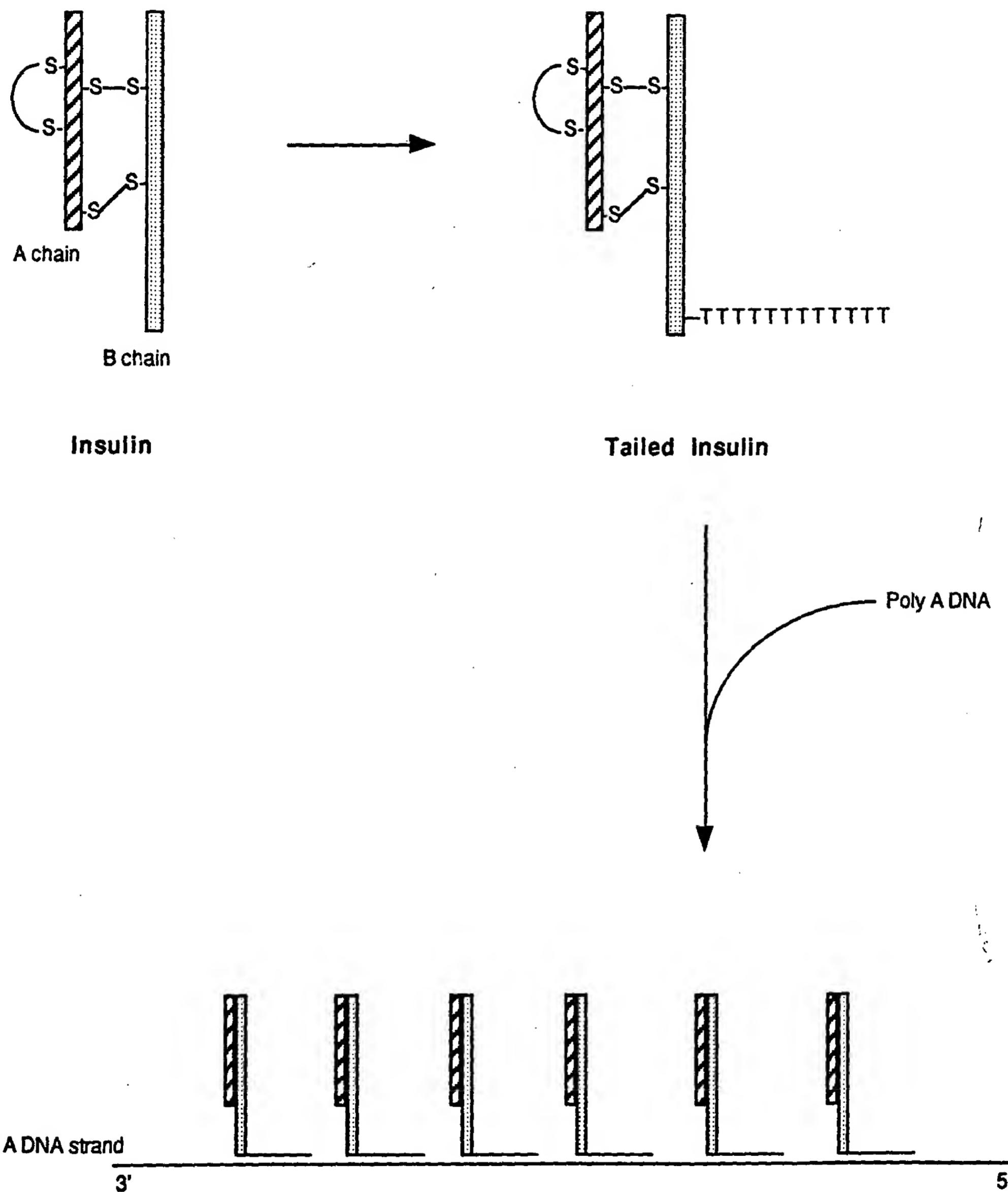
VI

**Figure 20**  
**Continuation of Synthetic Steps**

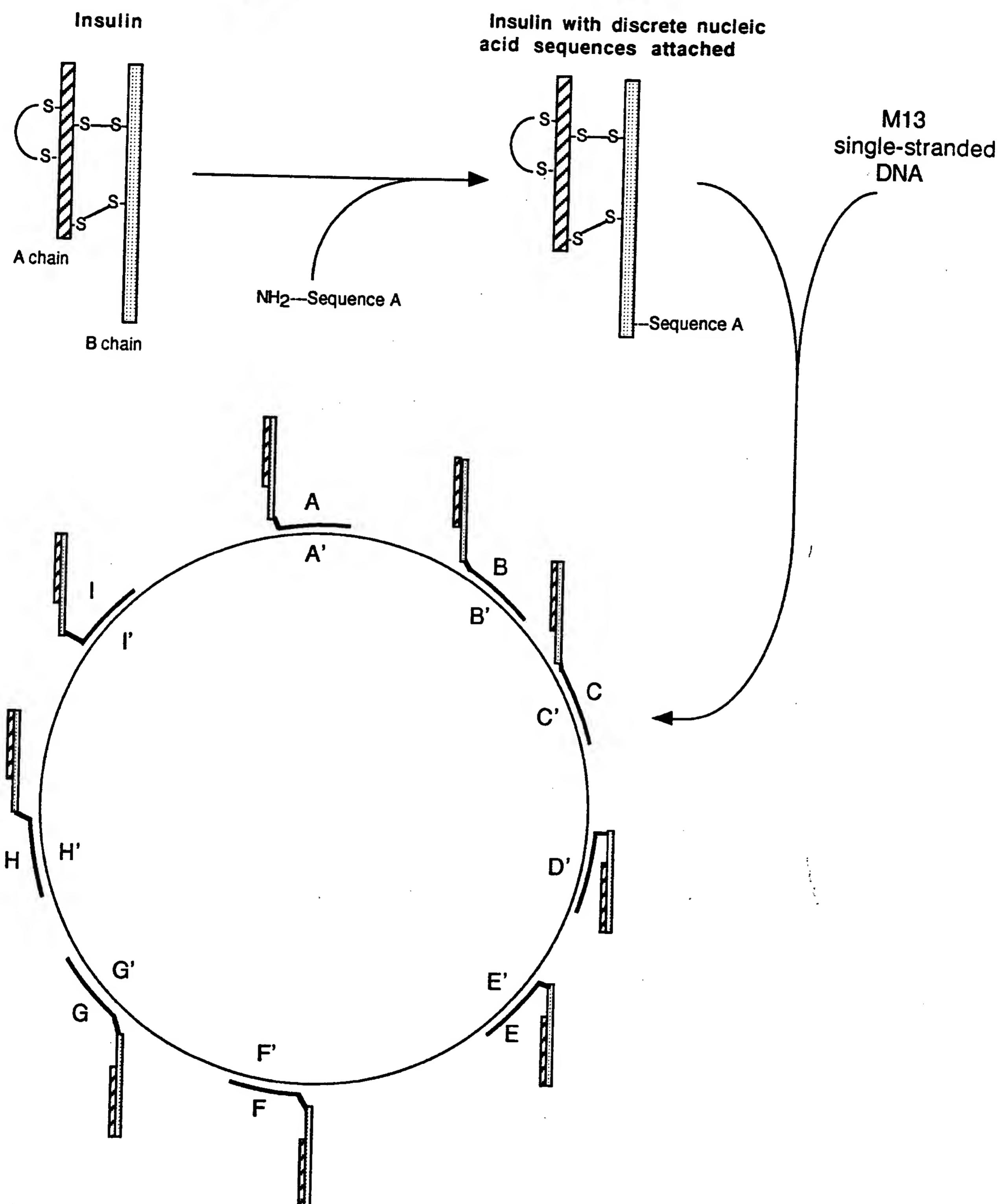
**Figure 21**

Enhanced Binding of Antibodies to Antigens by Multimerization

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**Figure 22**  
High Affinity Multi-Insulin Soluble Complex

**Figure 23**

Multimerization of Insulin molecules by hybridization to discrete Sequences

Intron insertion site  
↓  
(A) - - - **TGCTCTCTAAGGGTCTACTC** - - -  
- - - **ACGAGAGATTCCCAGATGAG** - - -

## T7 RNA Polymerase Sequence

(B)

The diagram shows two horizontal lines representing DNA strands. The top strand has a bolded sequence: **CTCTAAGGTAAATAT**. An arrow labeled "Splice Donor Site" points to the first "T" in "TATAT". The bottom strand has a bolded sequence: **GAGATTCCATTATA**. An arrow labeled "Splice Acceptor site" points to the last "T" in "TATA". Both strands have dashed lines extending from their ends.

## SV40 Intron Sequence

(C) **TGCTCTCTAAGGTAAATAT** - - - - - - - **TGTATTTAGGGTCTACTC** - - -  
**ACGAGAGATTCCATTATA** - - - - - - - **ACATAAAATCCAGATGAG** - - -

## Insertion of SV40 Intron into polymerase coding sequence

(D) **Splice Donor Site** ↓ **Splice Acceptor site** ↓  
 -----UGCUCUCUAAGGUAAAUAU----- UGUAUUUUAGGGGUCUACUC-----

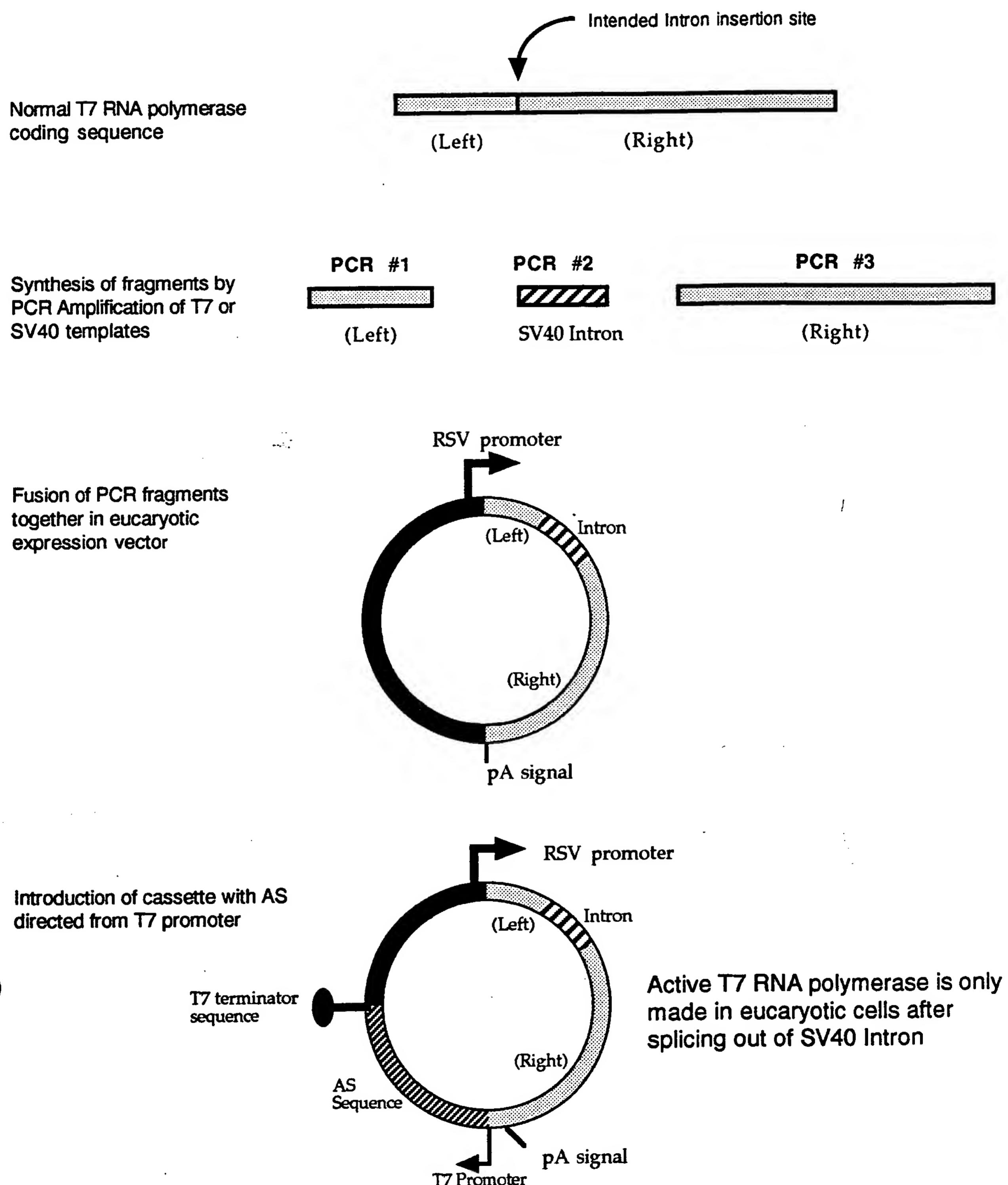
## mRNA transcript containing intron

(E) - - - **UGCUCUCUAAGGGUCUACUC** - - -

## mRNA transcript after splicing has normal T7 Sequence

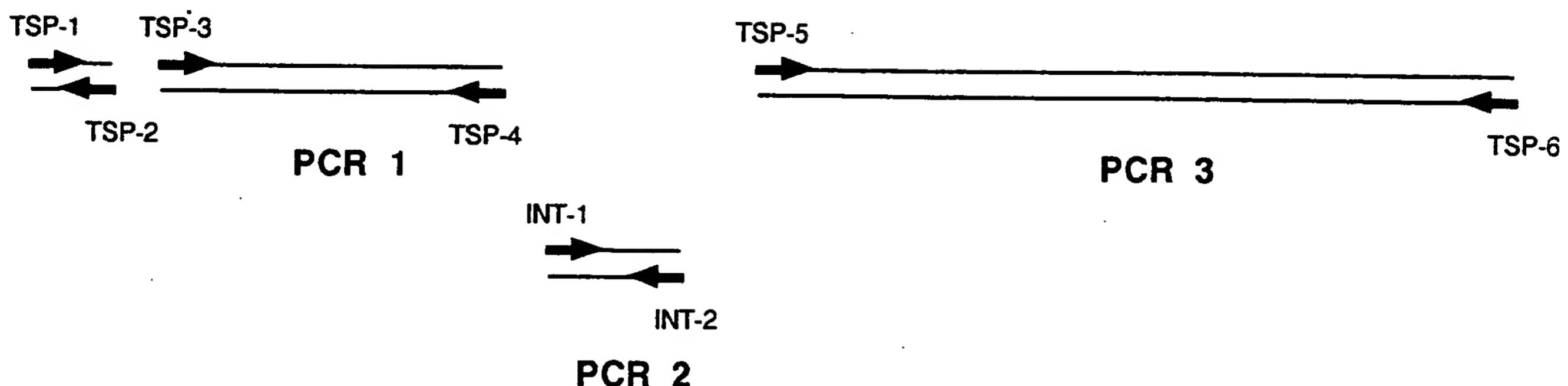
# **Figure 24**

## **Fusion of Intron into T7 RNA Polymerase Coding Sequence**



**Figure 25**  
**Construction of T7 Expression Vector**

### A) Synthesis of pieces



### B) Oligomers used for synthesis

TSP-1	GGA ATT CGT CTC GAG CTC TGA TCA CCA CCA TGG ACA CGA TTA ACA TCG C
TSP-2	GAC TAG TTG GTC TCG TCT CTT TTT TGG AGG AGT GTC GTT CTT AGC GAT GTT AAT C
TSP-3	GGA ATT CGT CTC GGA GAA AGG TAA AAT TCT CTG ACA TCG AAC TGG C
TSP-4	GAC TAG TGG TCT CCC CTT AGA GAG CAT GTC AGC
TSP-5	GGA ATT CGG TCT CGG GTC TAC TCG GTG GCG AGG
TSP-6	GAC TAG TCG TTA CGC GAA CGC AAA GTC
INT-1	GGA ATT CGT CTC TAA GGT AAA TAT AAA ATT TTT AAG
INT-2	GAC TAG TCG TCT CTG ACC CTA AAA TAC ACA AAC AAT TAG A

**Figure 26**  
**Synthesis of Pieces for Construction of**  
**T7 RNA Polymerase with Intron**

# Formation of Nuclear Localisation Signal by Fusion of TSP1/TSP2 Product to Clone with PCR #1 product

## Annealing of TSP1 with TSP2

TSP1

5' GG AAT TCA TCT CGA ACT CGT CGA ATC ACC ATG GAC ATT AAC GCT AAC GAC ACT CCT CCA AAA AAG AGA CGA GAC OAA CTA GTC  
 3' CC TTA AGC AGA GCT CGA GAC GTA TGG TAG CGA TAC CTG TGC TAA TAG CGA TTC TGG CTG TGA GGT TCT GCT CGT GTT GAT CAG  
 TSP2

## Extension of TSP1/TSP2 by polymerase

5' GG AAT TCG TCT CGA ACT CGT CGA ATC ACC ATG GAC ATT AAC GCT AAC GAC ACT CCT CCA AAA AAG AGA CGA GAC OAA CTA GTC  
 3' CC TTA AGC AGA GCT CGA GAC GTA TGG TAG CGA TAC CTG TGC TAA TAG CGA TTC TGG CTG TGA GGT TCT GCT CGT GTT GAT CAG  
 Bsa I

## Digestion of TSP1/TSP2 product with Bsa I

5' GG AAT TCG TCT CGA GCT CGT CGA ATC ACC ATG GAC ATT AAC GCT AAC GAC ACT CCT CCA AAA AAG AGA CGA GAC OAA CTA GTC  
 3' CC TTA AGC AGA GCT CGA GAC GTA TGG TAG CGA TAC CTG TGC TAA TAG CGA TTC TGG CTG TGA GGT TCT GCT CGT GTT GAT CAG  
 PCR

## Digestion of PCR #1 clone (pL-1) with BsmB I

Bsm B1  
 5' GGA ATT CGT CRC G  
 CCT TAA GCA GAG CCTCT  
 GAGA AAG GTA AAA TTC TCT GAC ATC GAA CGT GC-----  
 TTC CAT TTT AAG AGA CGT TAG CTT GAC CG-----

## Ligation of Bsa I digested TS1/TS2 product to BsmB I digested PCR#1 clone

5' GG AAT TCG TCT CGA GCT CGT CGA ATC ACC ATG GAC ATT AAC GAC ACT CCT CCA AAA AAG AGA CGA GAC OAA CTA GTC  
 3' CC TTA AGC AGA GCT CGA GAC GTA TGG TAG CGA TAC CTG TGC TAA TAG CGA TTC TGG CTG TGA GGT TCT GCT CGT GTT AAG  
 TCT GAC ATC GAA CGT GC-----  
 AGA CGT TAG CTT GAC CG-----

**Comparison of the 5' ends of the Nucleotide Sequences of Wild Type  
and Modified T7 RNA Polymerase**

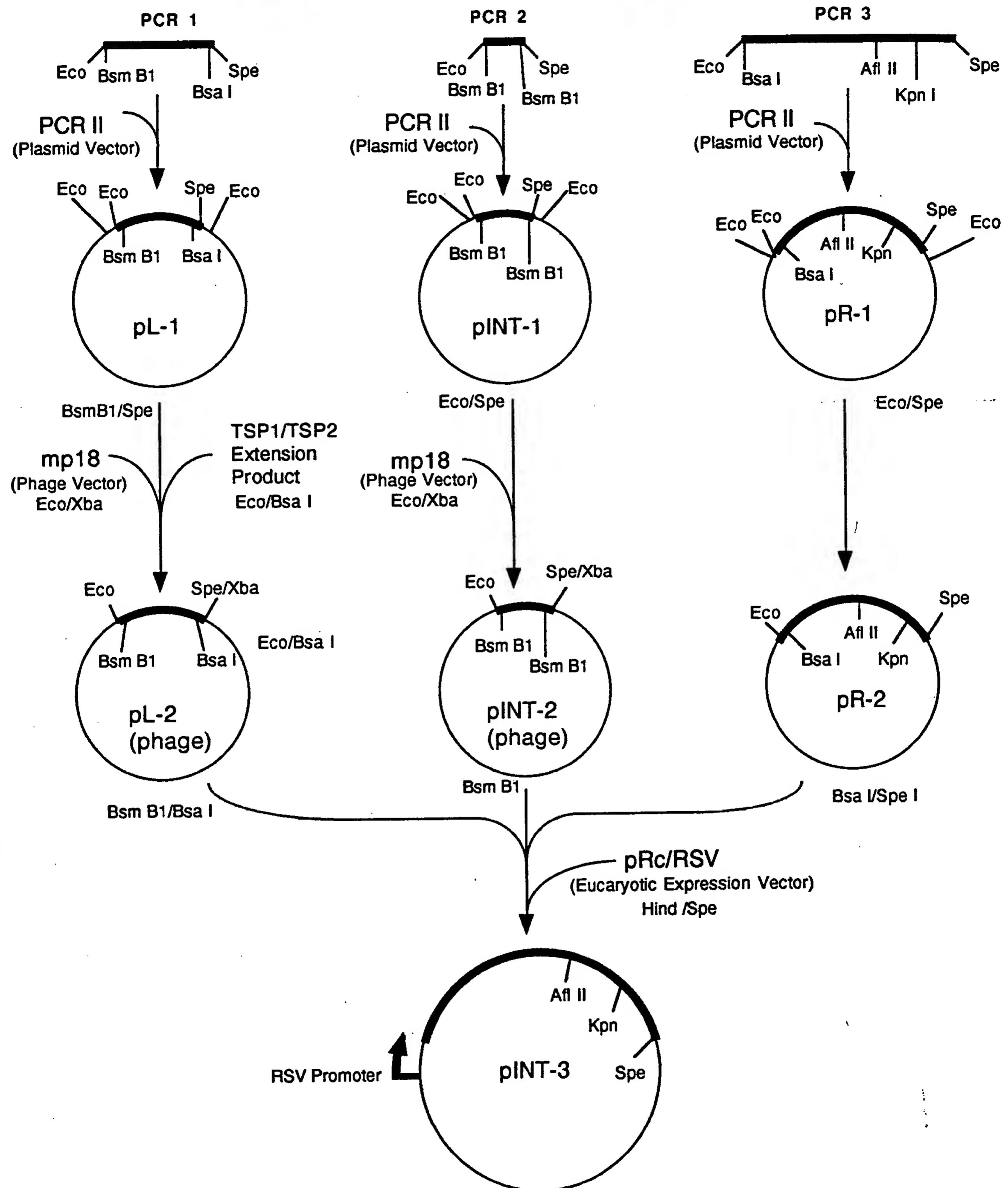
**Wild Type T7 nucleic and amino acid sequence**

ATG	GAC	ACG	ATT	AAC	ATC	GCT	AAG	AAC	TTC	TCT	GAC	ATC	GAA	CTG	GC-----	
TAC	CTG	TGC	TAA	TTC	TGA	TGG	CGA	TTC	TTC	CTG	AGA	CTG	TAG	CTT	GAC	CG-----
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

**Modified T7 nucleic and amino acid sequence  
with Nuclear Localisation Signal (NLS) insertion**

ATG	GAC	ACG	ATT	AAC	ATC	GCT	AAG	AAC	TTC	TCT	GAC	ATC	GAA	CTG	GC-----	
TAC	CTG	TGC	TAA	TTC	TGA	TGG	CGA	TTC	TTC	TCT	TTC	CAT	TTC	AAG	CTG	CG-----
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

**Figure 28**

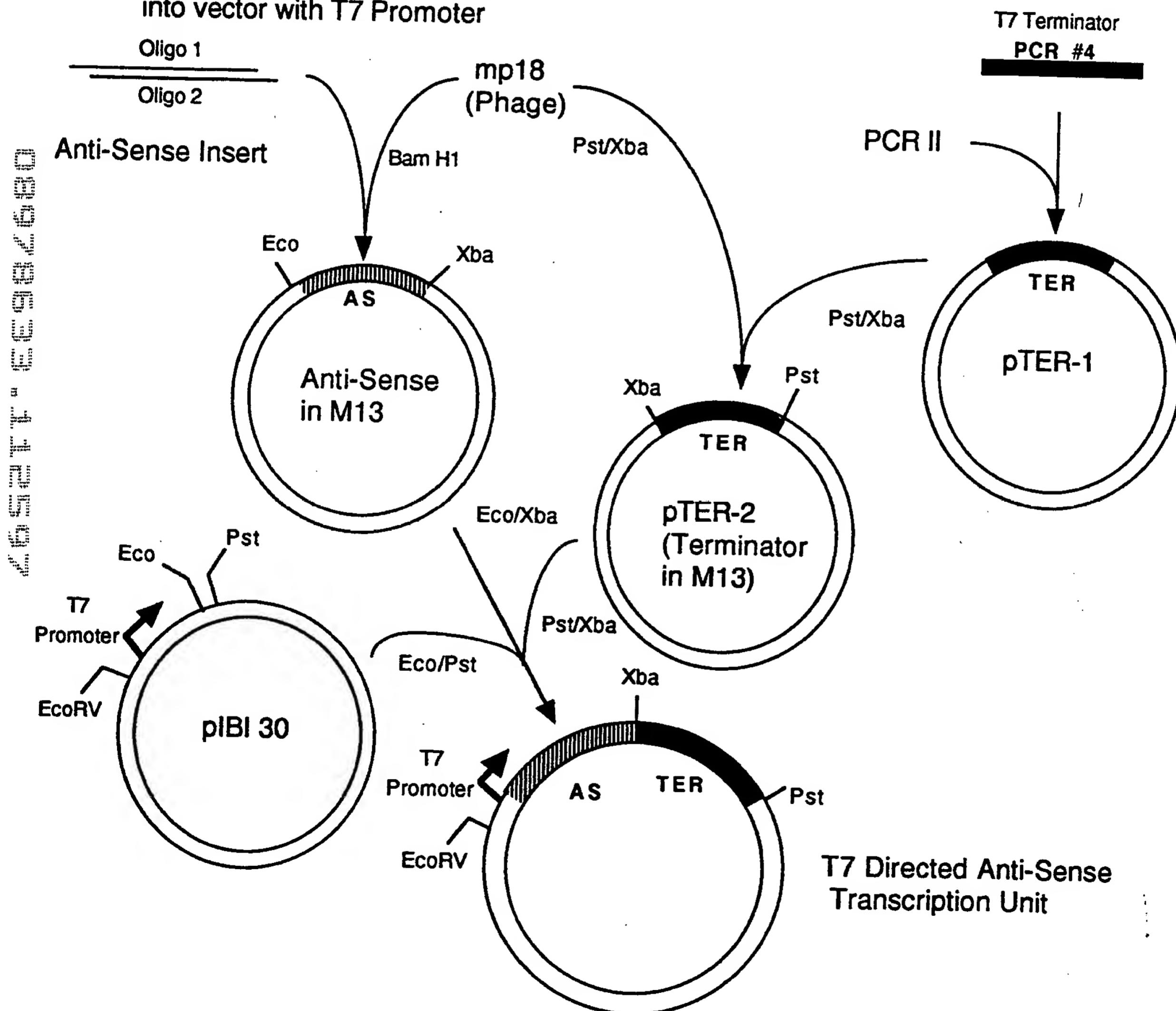
**Figur 29**

Fusion of PCR Pieces to Construct  
T7 RNA Polymerase with an Intron

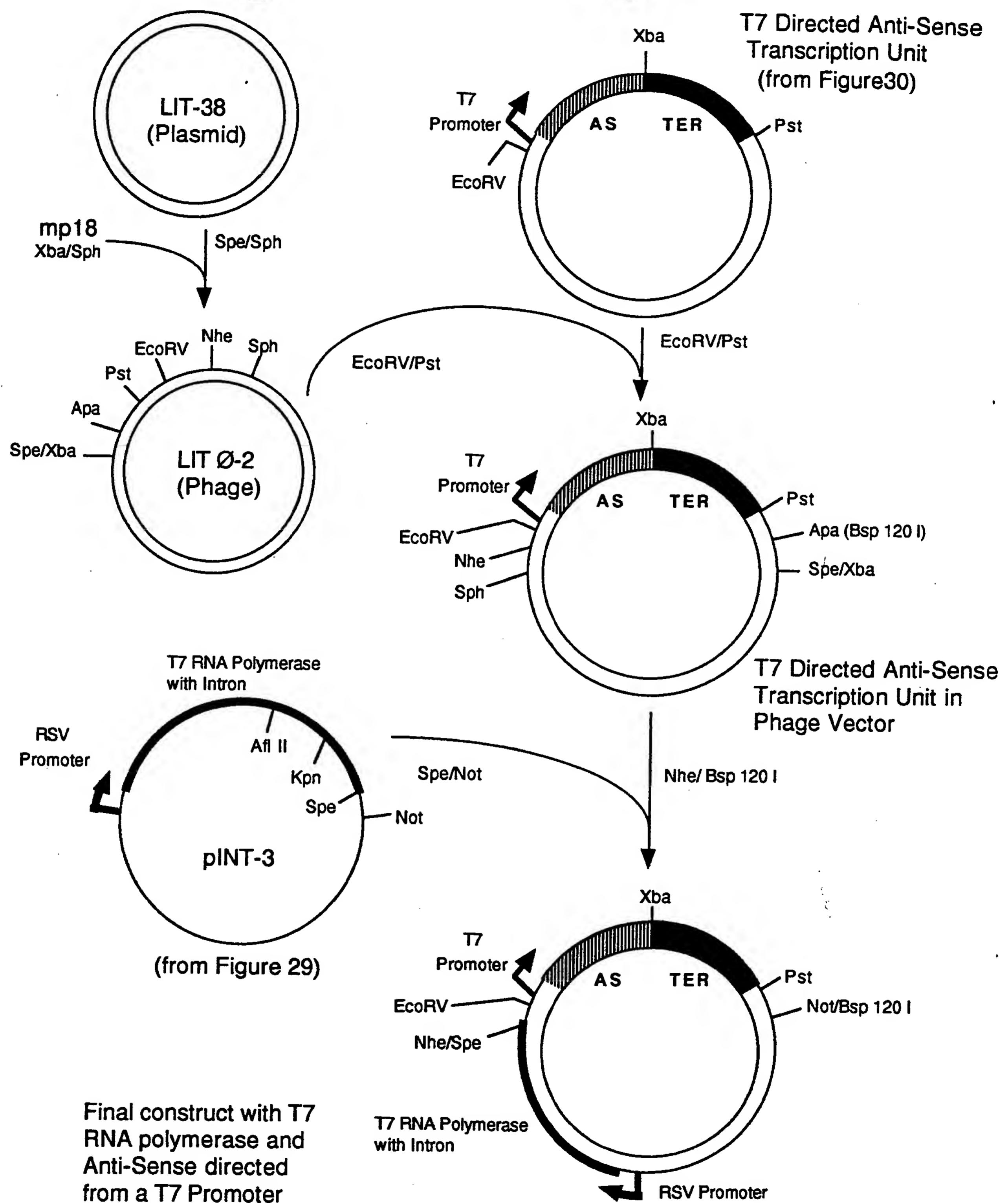
## (A) Oligomers

HTA-1 GAT CAT TAG ACC AGA TCT GAG CCT GGG AGC TCT CTG GCT AAC TAG GGA ACC CAC TGCTTA AGC CTC AAG  
 HTA-2 GAT CCT TGA GGC TTA AGC AGT GGG TTC CCT AGT TAG CCA GAG AGC TCC CAG GCT CAG ATC TGG TCT AAT  
 HTB-1 GAT CAC CTT AGG CTC TCC TAT GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA CCT CCT CAA G  
 HTB-2 GAT CCT TGA GGA GGT CTT CGT CGC TGT CTC CGC TTC CTG CCA TAG GAG AGC CTA AGG T  
 HTC-1 GAT CAT AGT GAA TAG AGT TAG GCA GGG ATA CTC ACC ATT ATC GTT TCA GAC CCA CCT CCC AG  
 HTC-2 GAT CCT GGG AGG TGG GTC TGA AAC GAT AAT GGT GAG TAT CCC TGC CTA ACT CTA TTC ACT AT  
 TER-1 AAT CTA GAG CTA ACA AAG CCC GAA AGG AAG  
 TER-2 TTC TGC AGA TAT AGT TCC TCC TTT CAG C

## (B) Cloning of AS and Terminator sequences into vector with T7 Promoter



**Figure 30**  
Insertion of Anti-Sense Sequences into  
T7 Directed Transcription Units

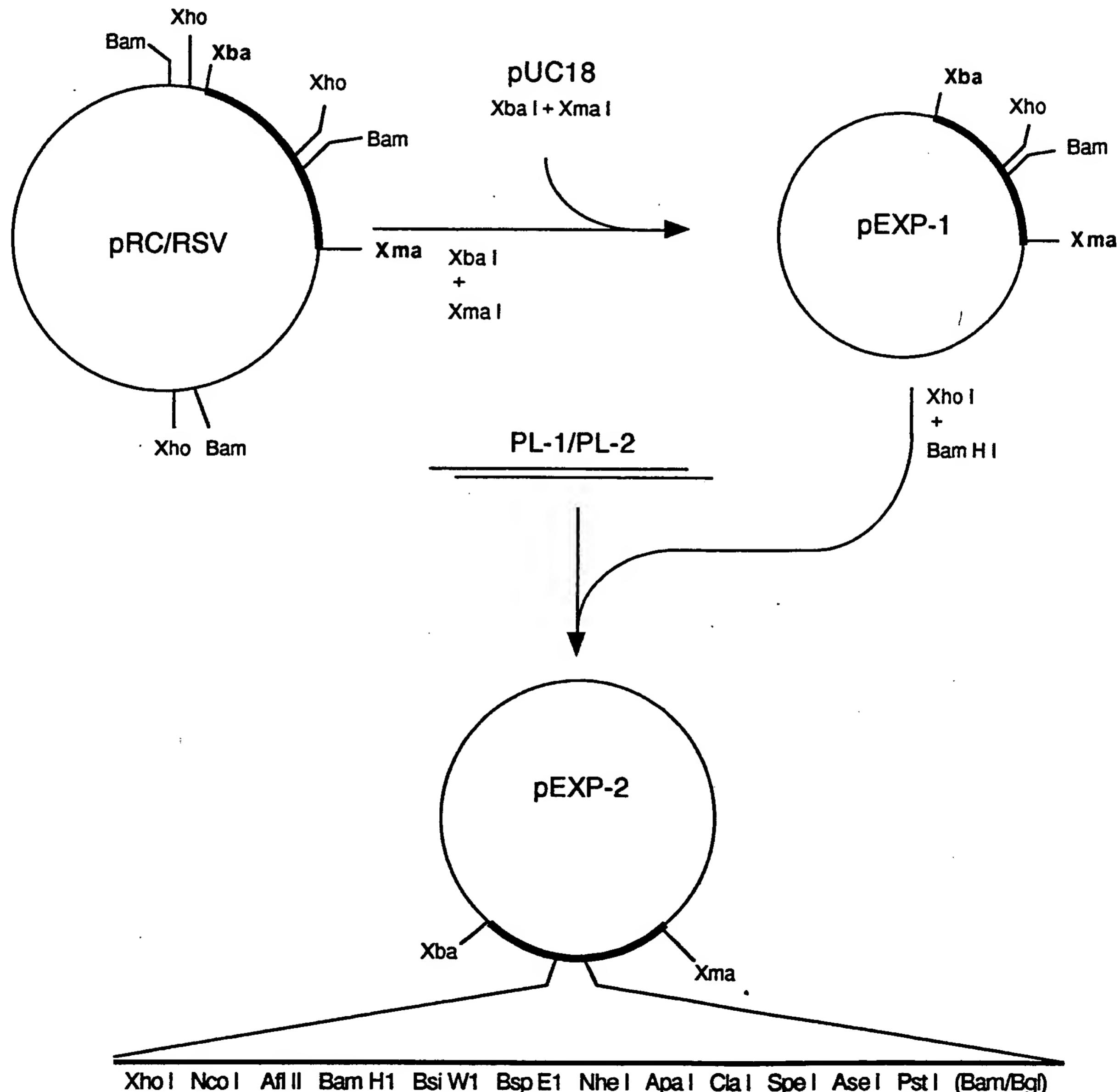
**Figure 31**

Construct with T7 RNA polymerase and Anti-Sense directed from a T7 Promoter

**A) Oligomers for introduction of T7 signals and polylinker**

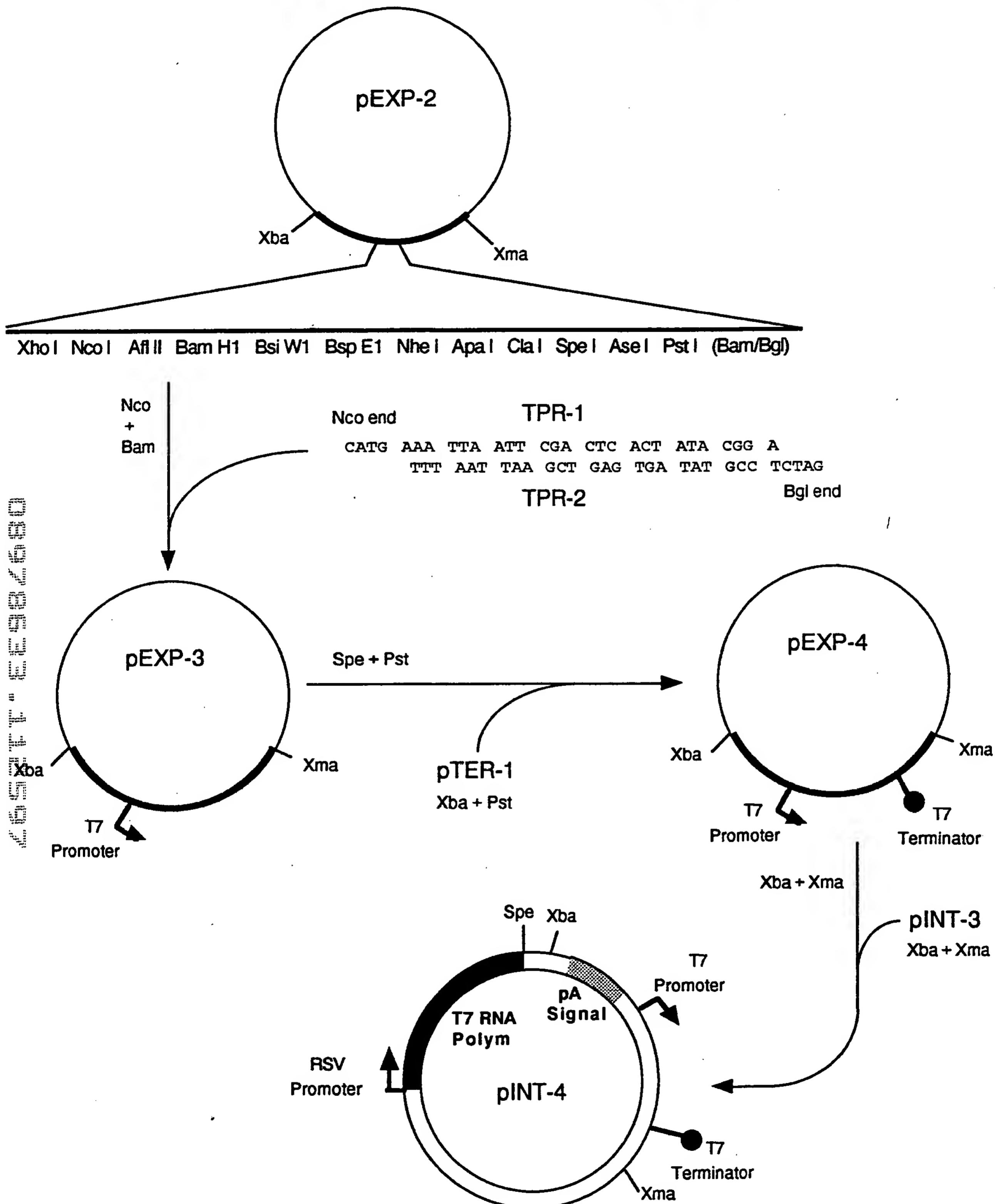
**PL-1**    TCG AGC CAT GGC TTA AGG ATC CGT ACG TCC GGA GCT AGC GGG CCC ATC GAT ACT  
            AGT TAA ATG CAG ATC T

**PL-2**    CTA GAG ATC TGC ATT TAA CTA GTA TCG ATG GGC CCG CTA GCT CCG GAC GTA CGG  
            ATC CTT AAG CCA TGG C

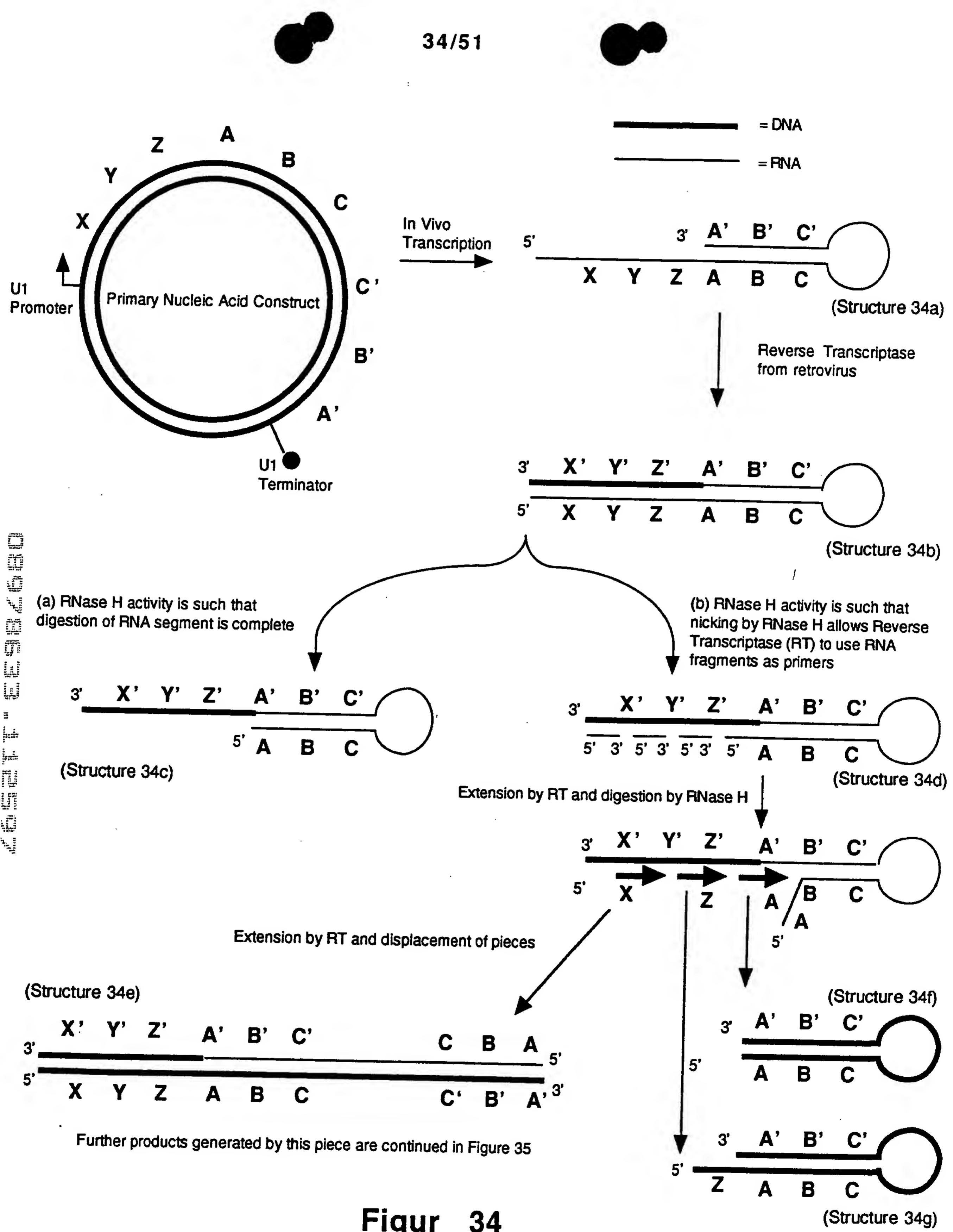


**Figure 32**

**Introduction of Poly-Linker for Creation of Protein Expression Vector**



**Figure 33**  
Final steps for construction of Expression Vector

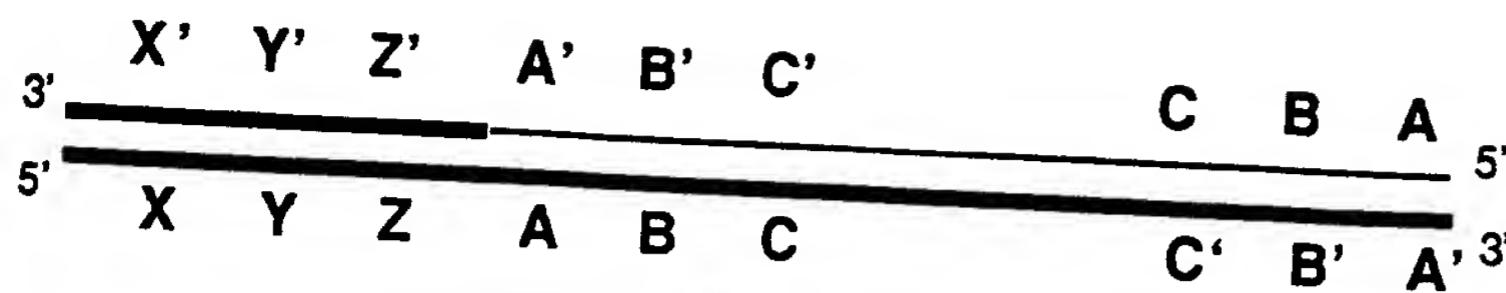


Figur 34

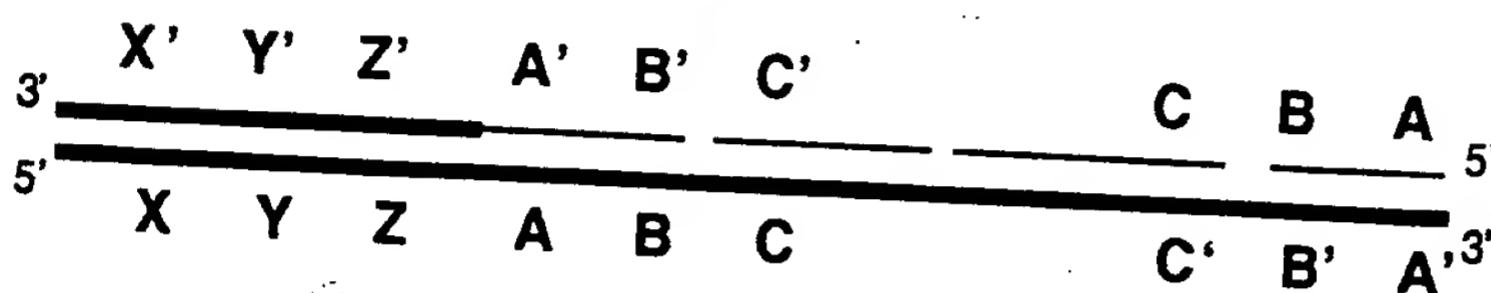
Construct that produces single-stranded Anti-Sense DNA

Continued from Figure 34

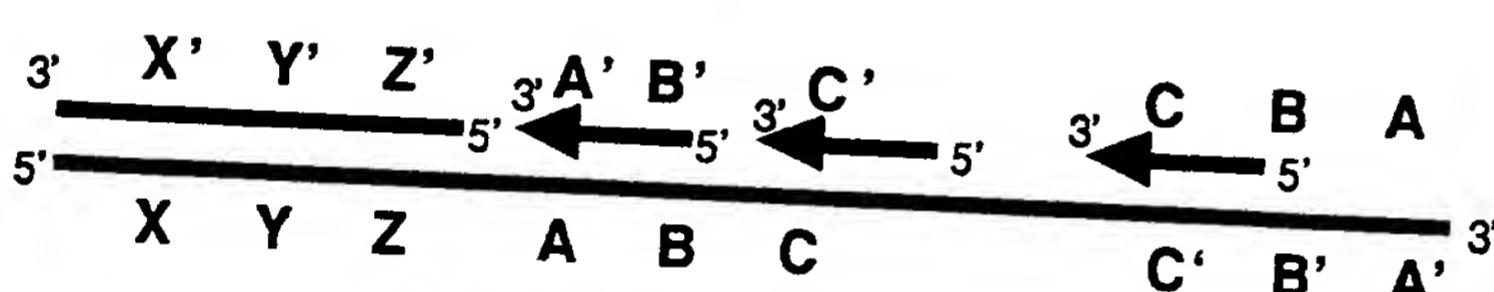
(Structure 34e)



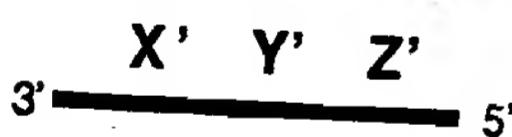
Nicking by RNase H



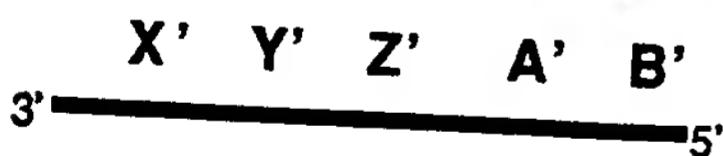
Extension by RT and digestion by RNase H



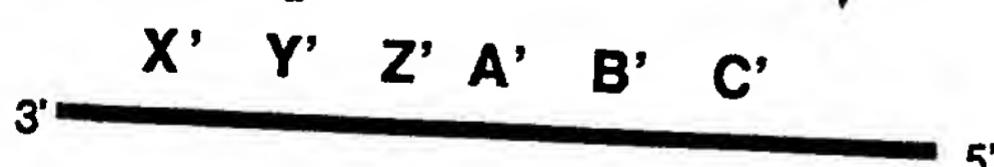
(Structure 35h)



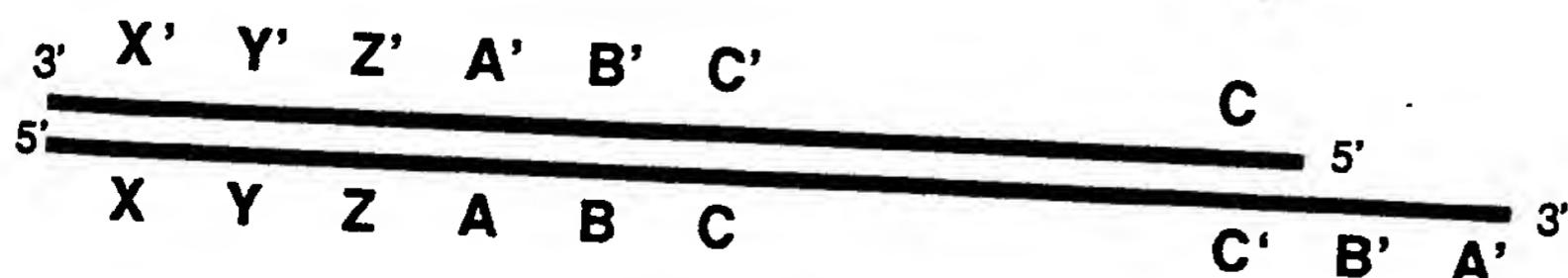
(Structure 35i)



(Structure 35j)

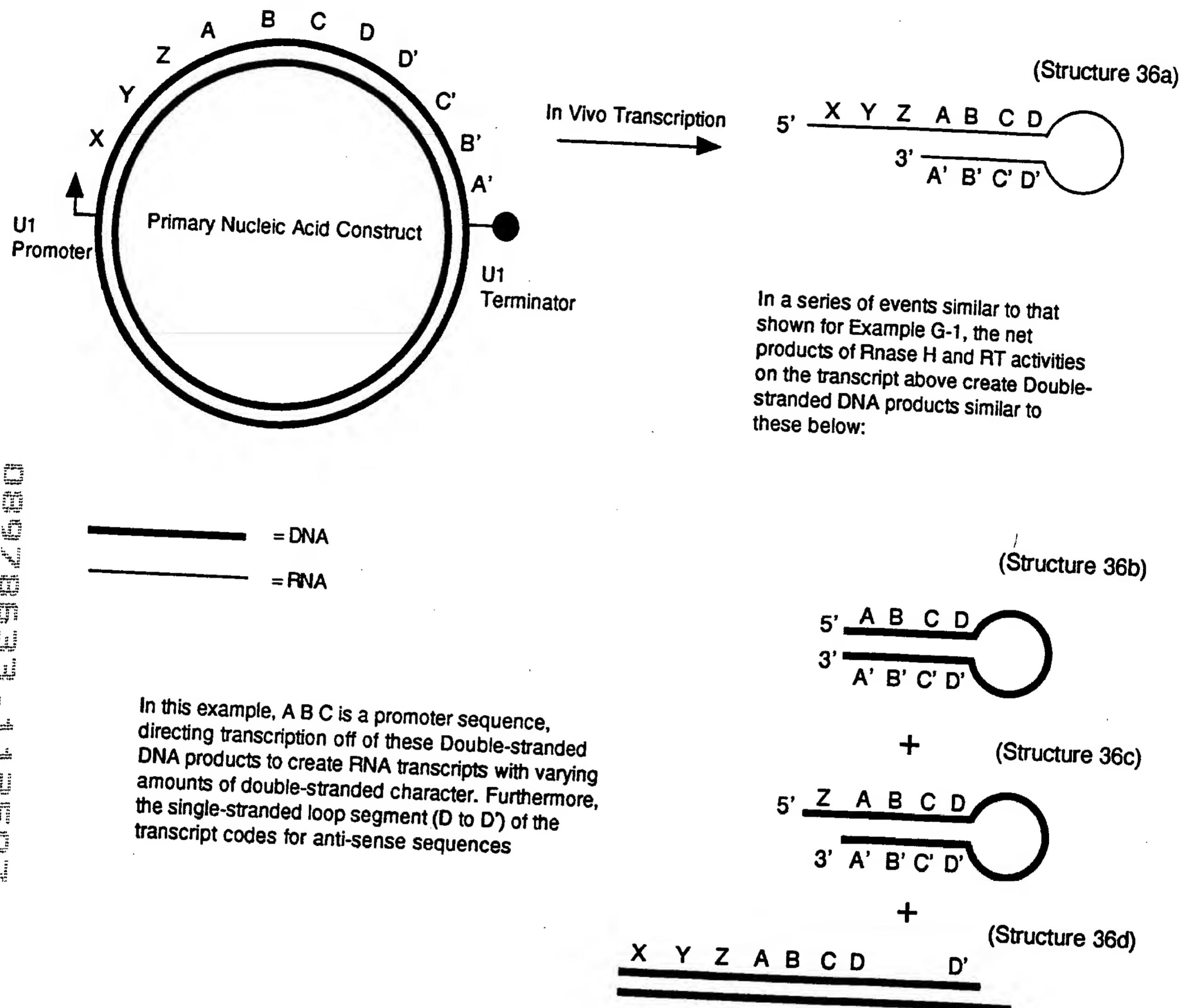


(Structure 35k)



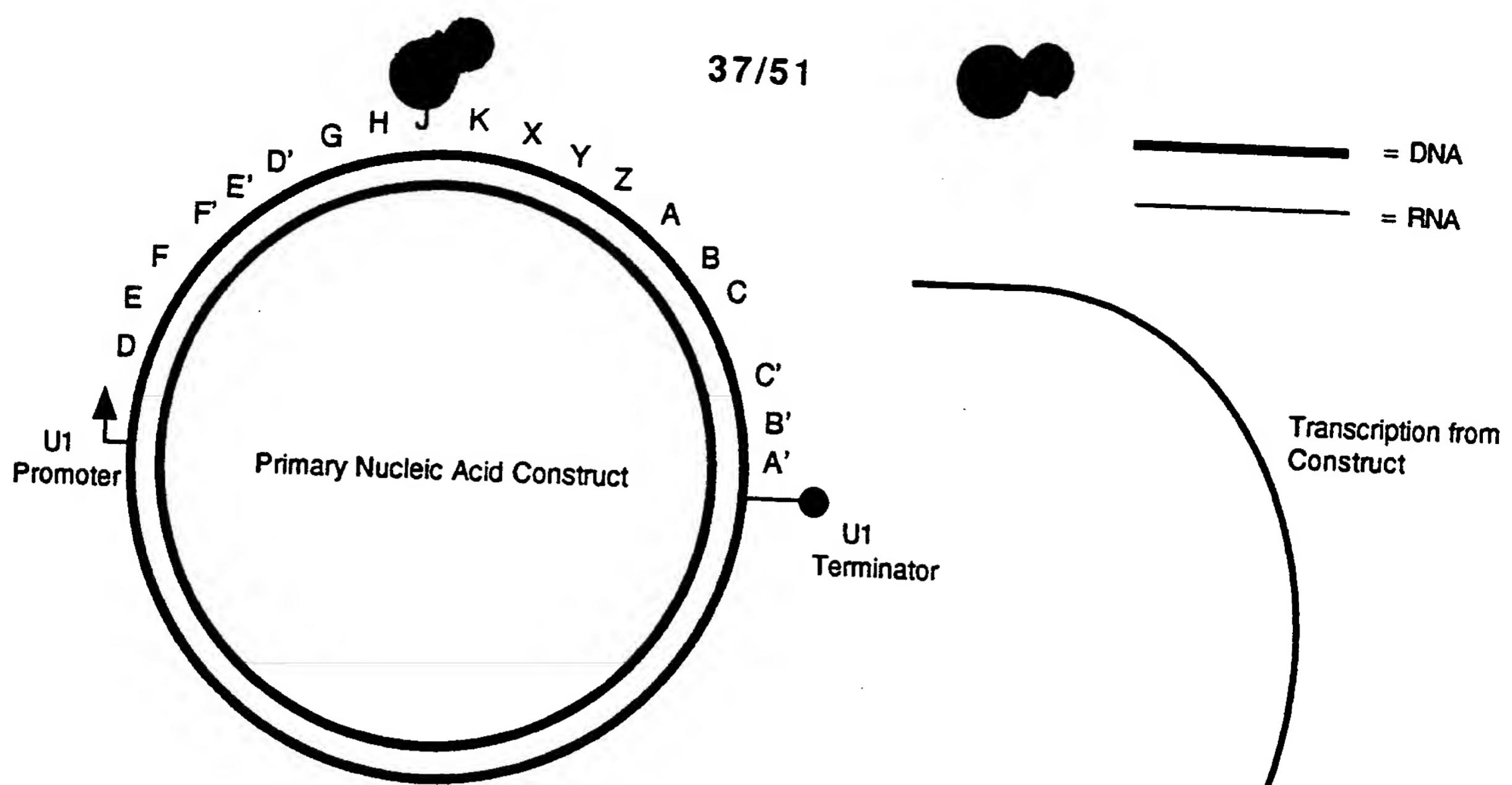
Extension by RT and displacement generates Single-Stranded DNA and a mostly Double-stranded DNA molecule

**Figure 35**  
Continuation of Process from Figure 34

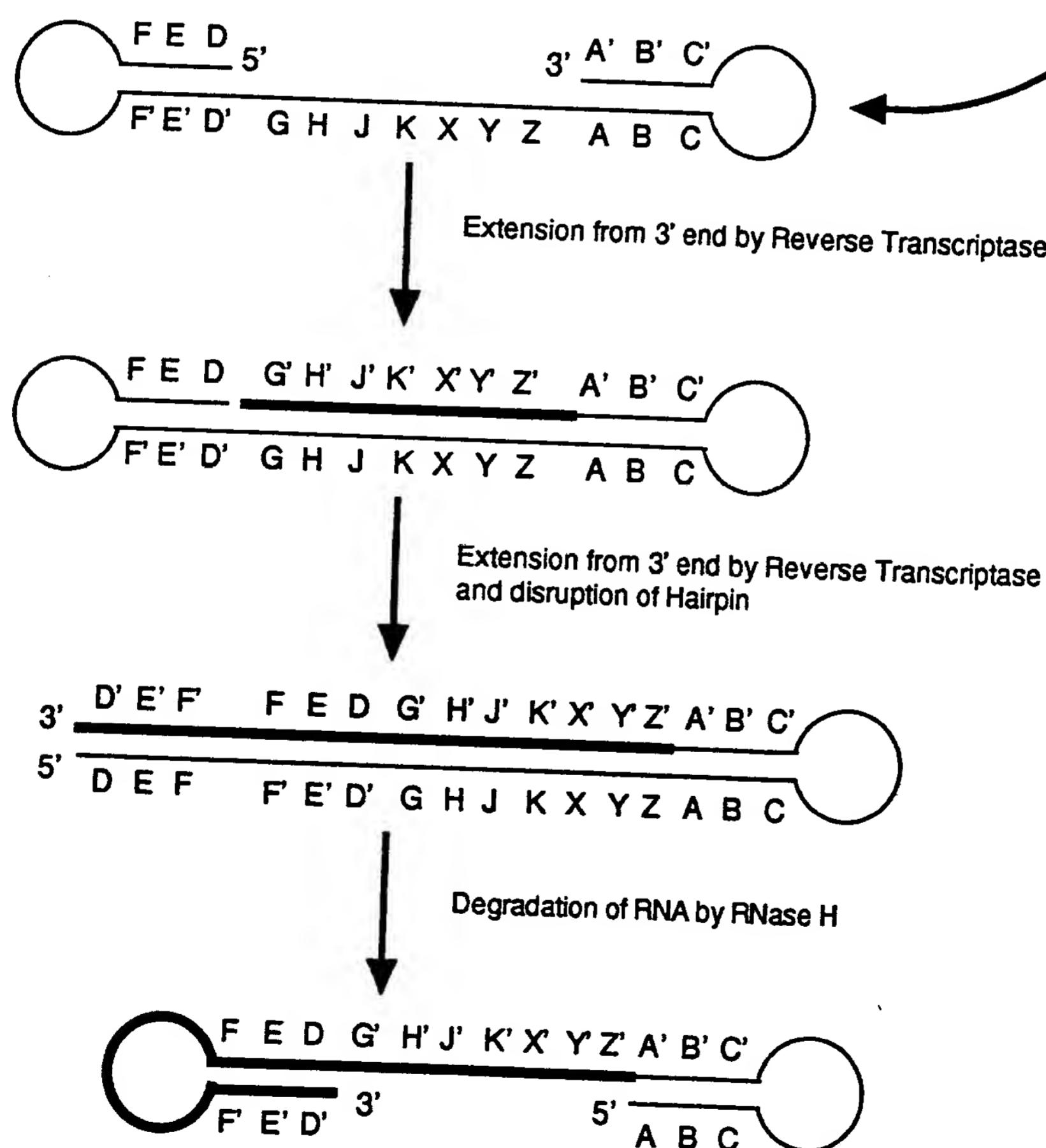


### Figure 36

Construct that produces RNA that is Reverse Transcribed to create Secondary DNA Constructs capable of directing transcription

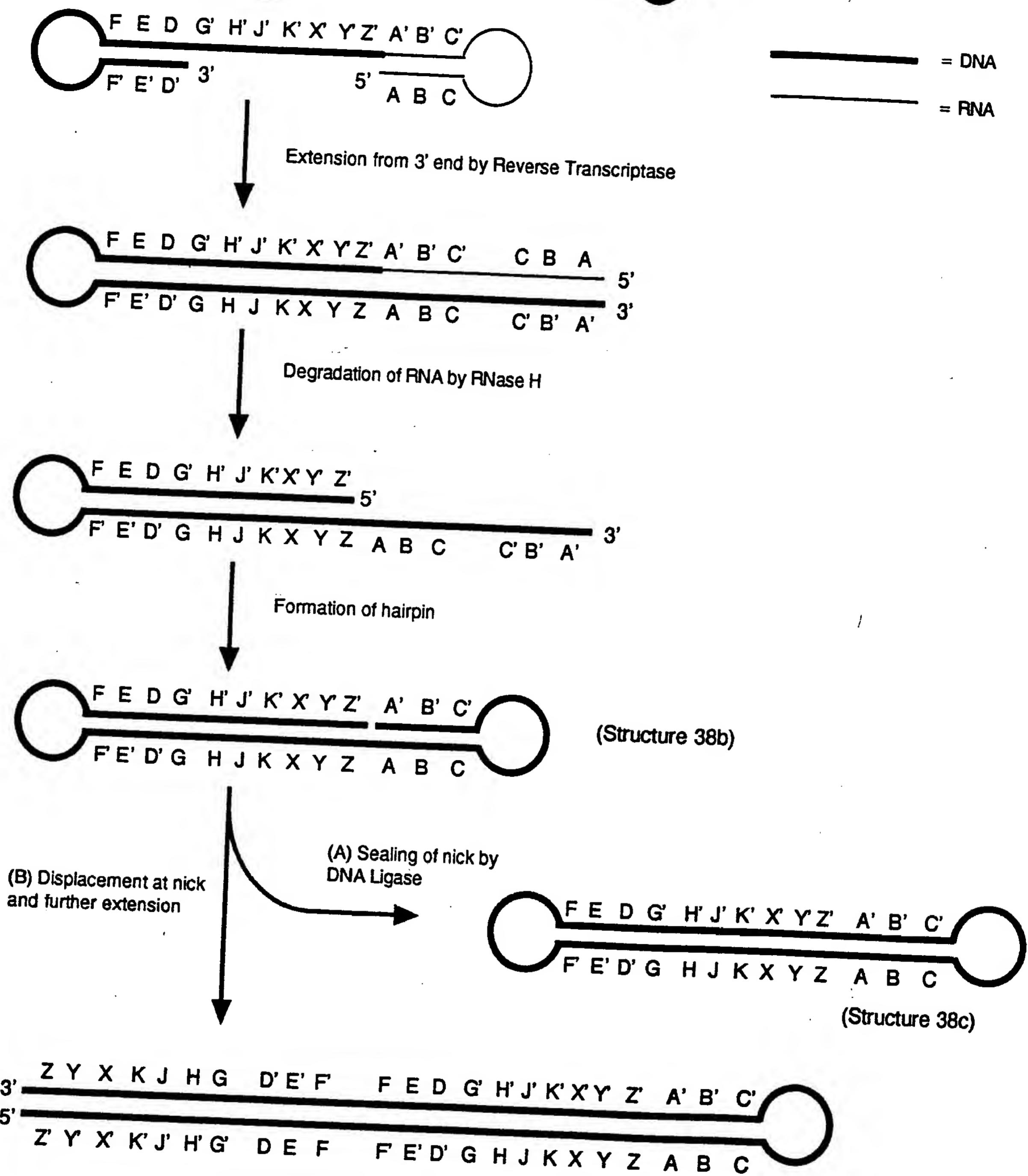


(Structure 37a)



(Continued in Figure 38)

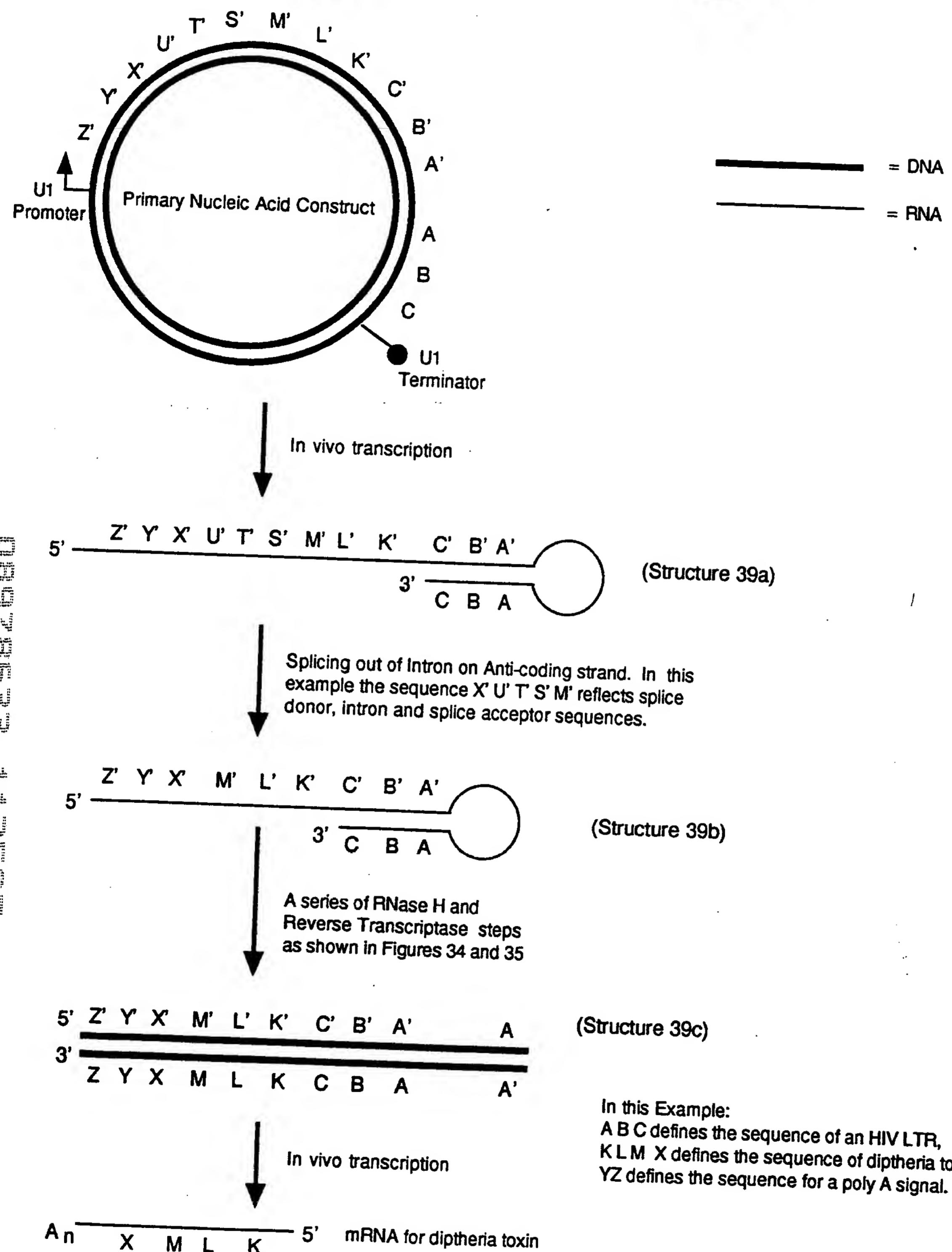
**Figure 37**  
Construct which Propagates a Double Hairpin Production Center



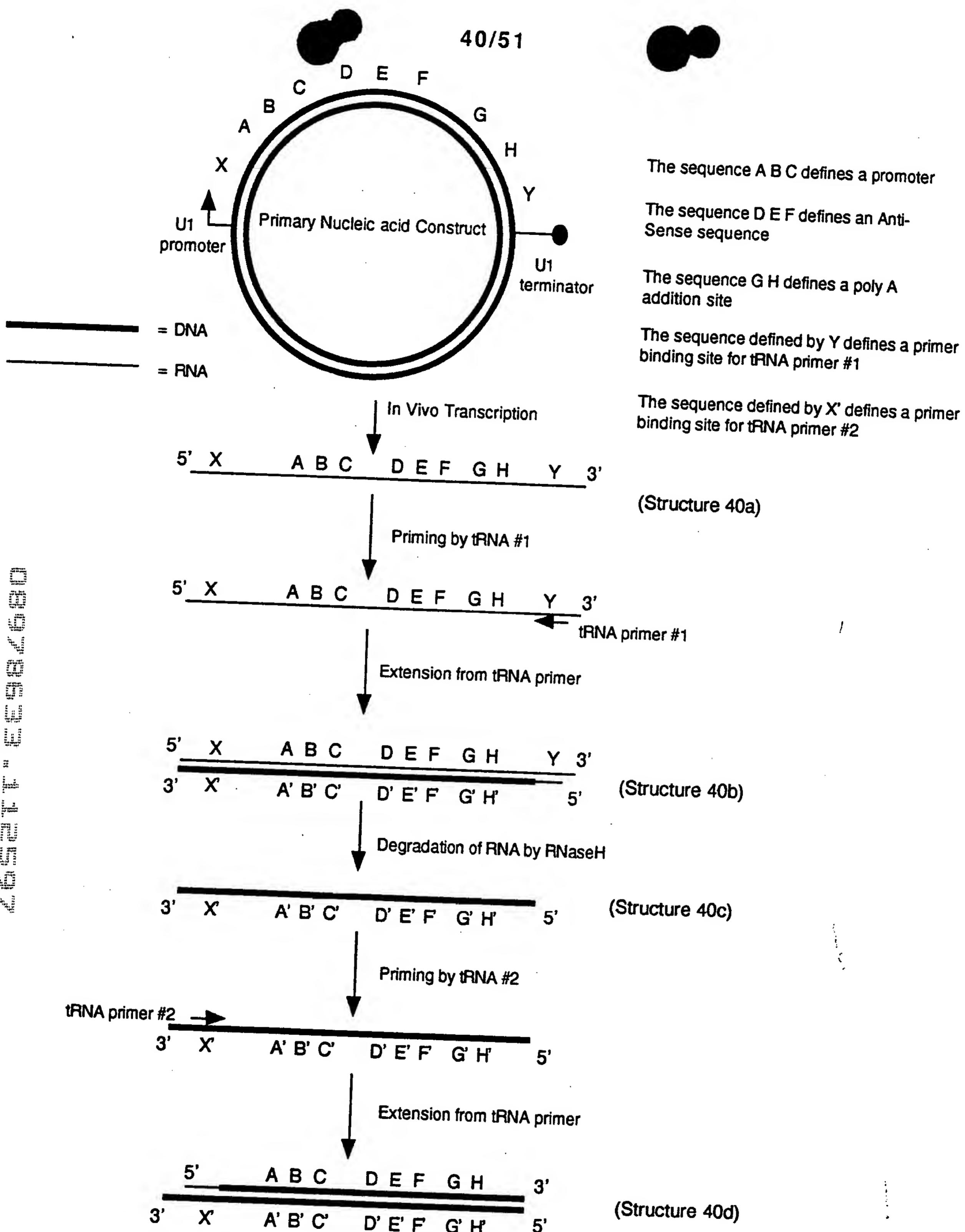
In this Example, the sequence F' E' D' is a promoter, the sequence G H J K is an Anti-Sense sequence and X Y Z is a Poly A signal

(Structure 38d)

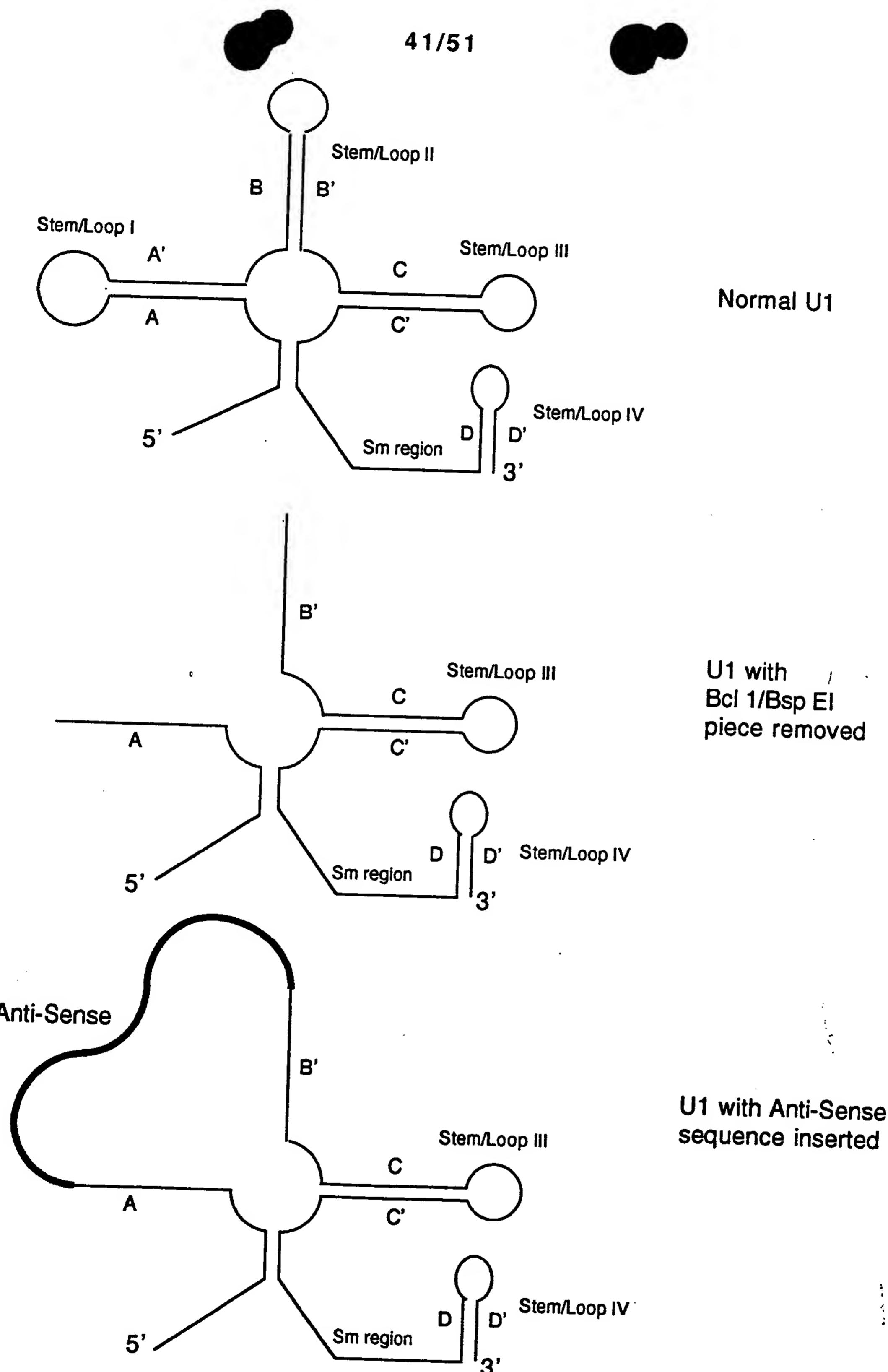
**Figure 38**  
Continuation of process from Figure 37



**Figure 39**  
Construct which propagates a Production Center capable of Inducible Suicide



**Figure 40**  
Use of tRNA primers to create a DNA construct  
for secondary production of transcripts

**Figure 41**

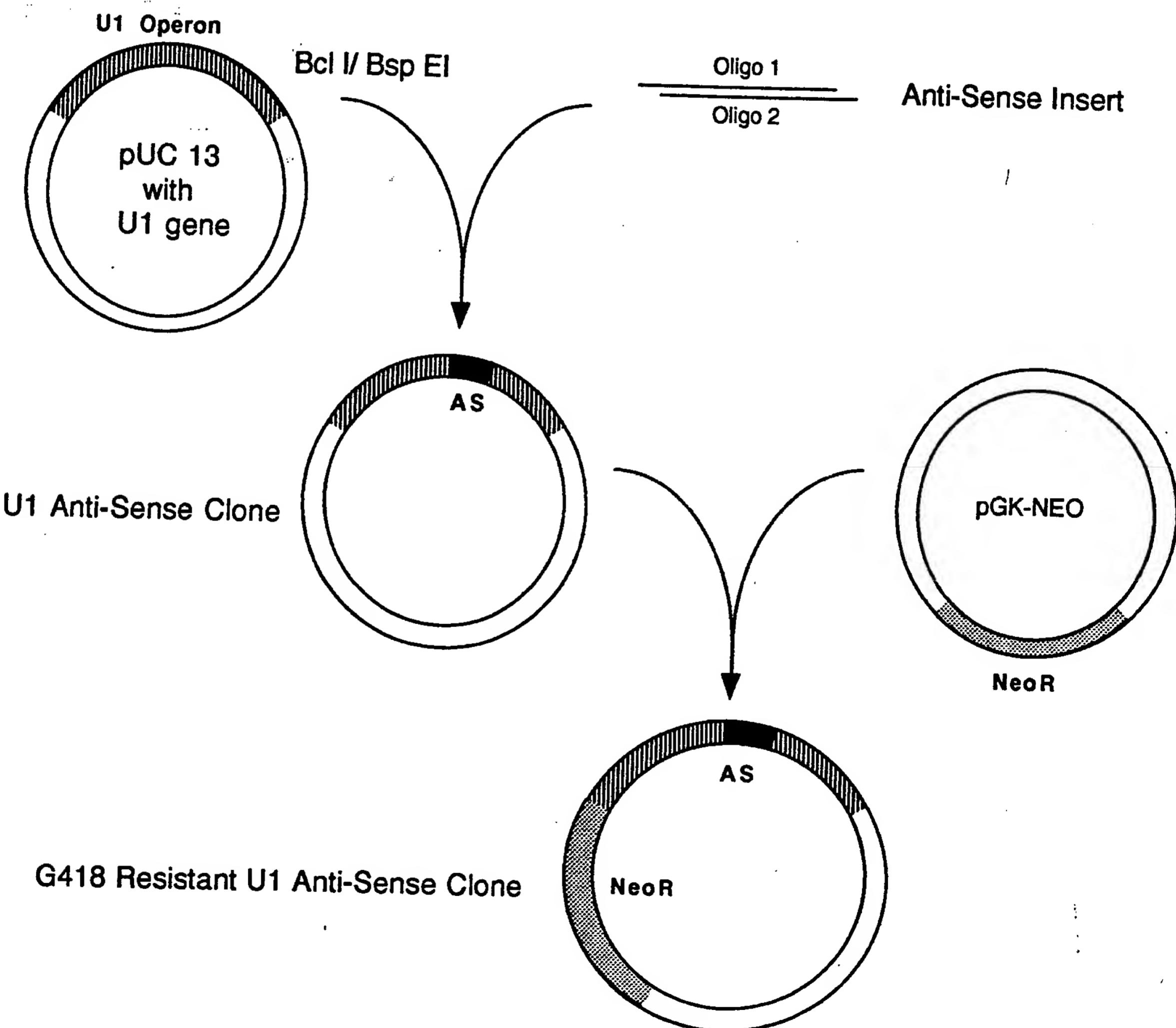
Excision of Sequences from U1 Transcript Region  
and Replacement with Novel Sequences

## (A) Anti-sense oligomers

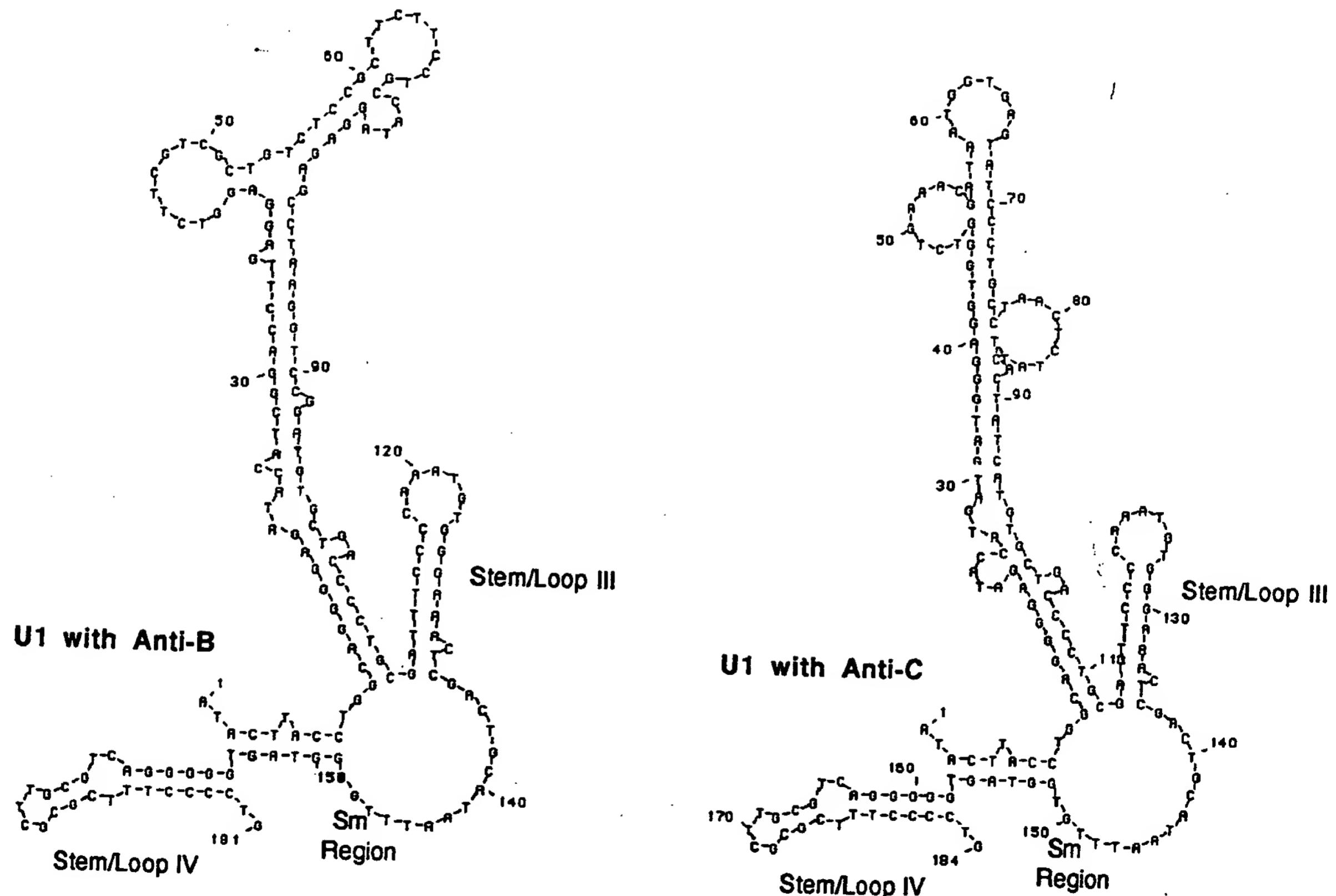
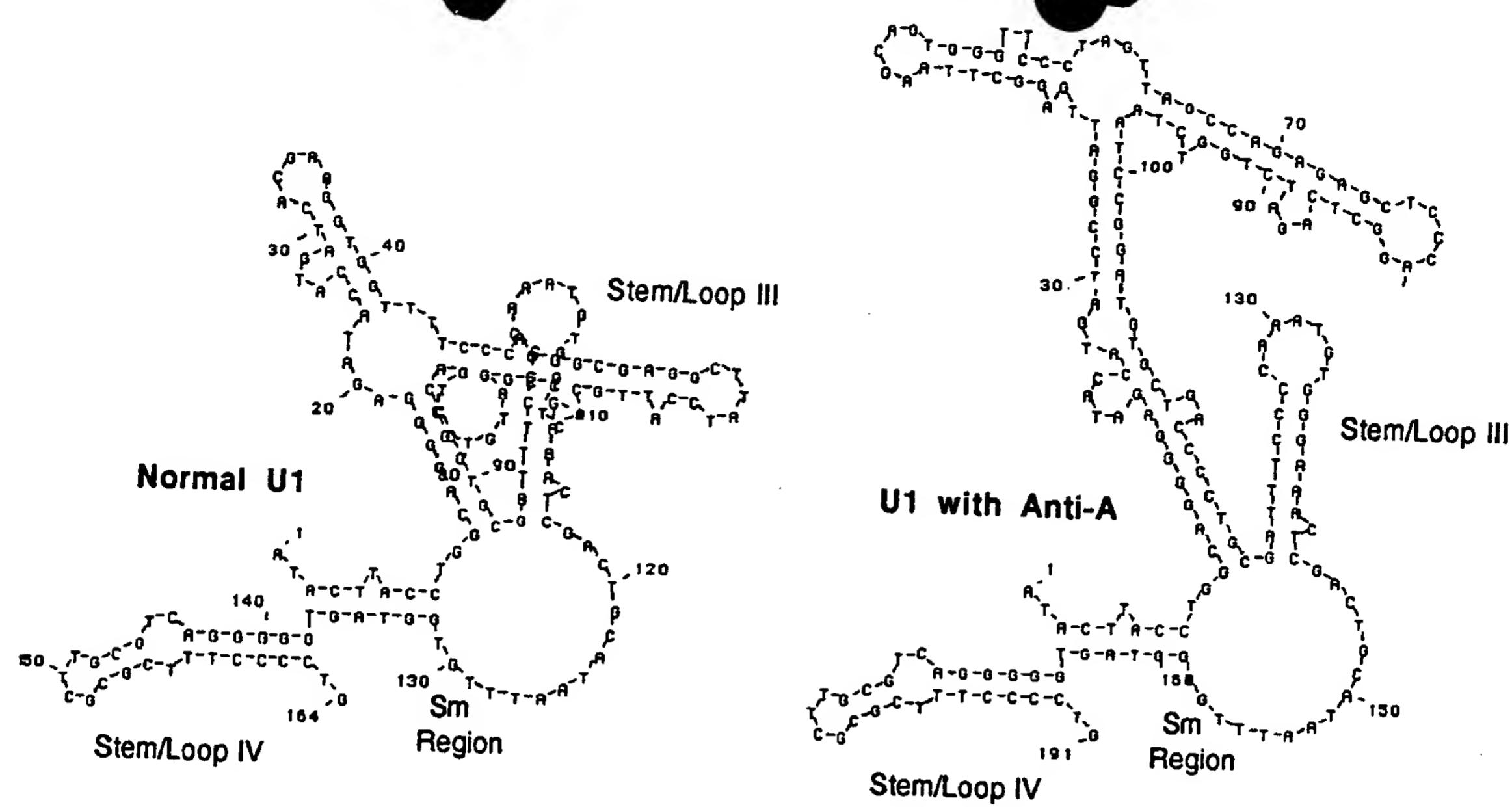
HVA-1 GAT CCG GAT TGA GGC TTA AGC AGT GGG TTC CCT AGT TAG CCA GAG AGC TCC CAG GCT CAG ATC TGG TCT AAT  
 HVA-2 CCG GAT TAG ACC AGA TCT GAG CCT GGG AGC TCT CTG GCT AAC TAG GGA ACC CAC TGC TTA AGC CTC AAT CCG  
 HVB-1 GAT CCG GAC CTT GAG GAG GTC TTC GTC GCT GTC TCC GCT TCT TCC TGC CAT AGG AGA GCC TAA GGT  
 HVB-2 CCG GAC CTT AGG CTC TCC TAT GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA CCT CCT CAA GGT CCG  
 HVC-1 GAT CCG GAT GGG AGG TGG GTC TGA AAC GAT AAT GGT GAG TAT CCC TGC CTA ACT CTA TTC ACT AT  
 HVC-2 CCG GAT AGT GAA TAG AGT TAG GCA GGG ATA CTC ACC ATT ATC GTT TCA GAC CCA CCT CCC ATC CG  
 HVD-1 GAT CAG CAT GCC TGC AGG TCG ACT CTA GAC CCG GGT ACC GAG CTC GCC CTA TAG TGA GT C GT A TT A T  
 HVD-2 CCG GAT AAT ACG ACT CAC TAT AGG GCG AGC TCG GTA CCC GGG TCT AGA GTC GAC CTG CAG GCA TG C T

## (B) Replacement of U1 sequences with HIV Anti-sense sequences

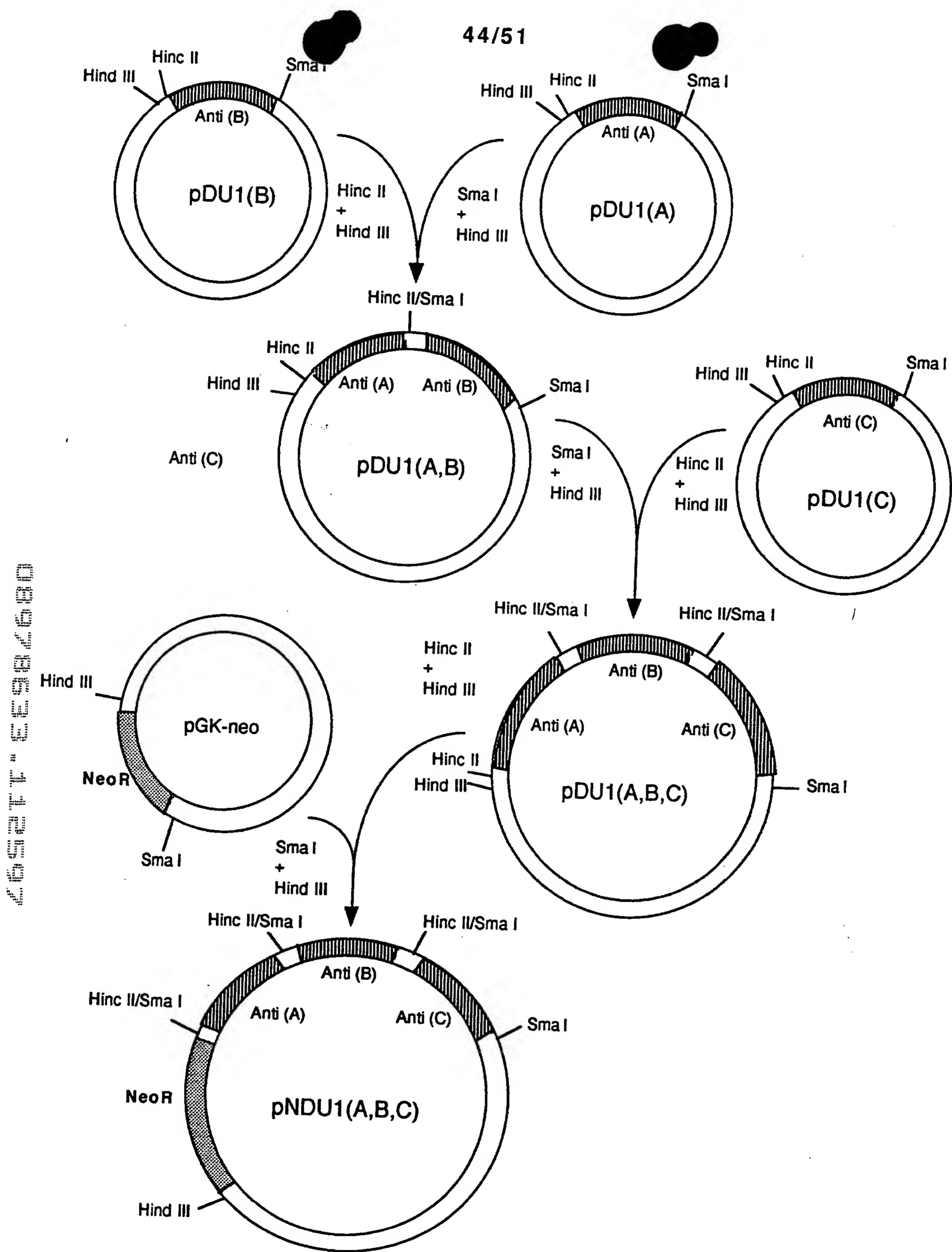
00000000000000000000000000000000



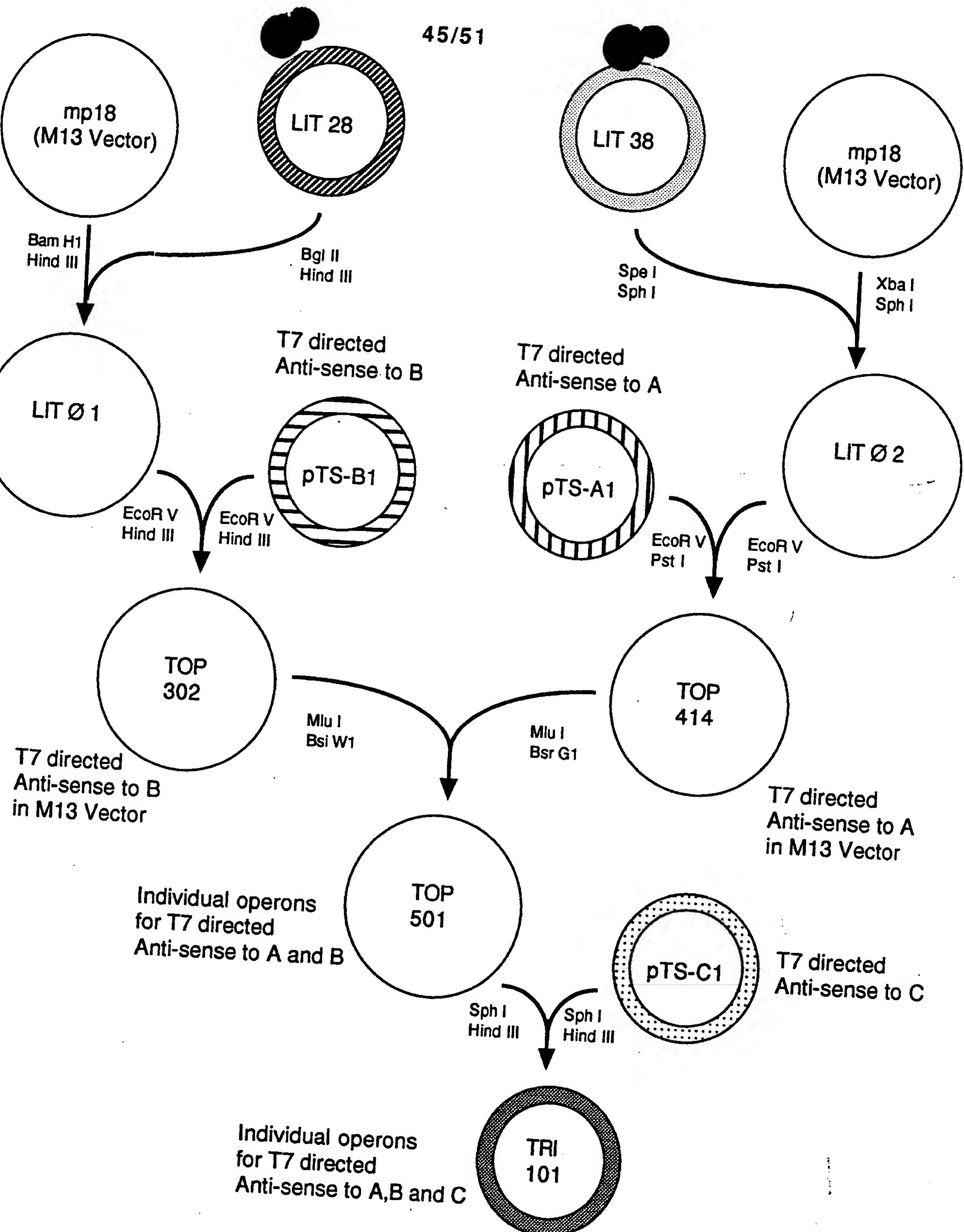
**Figure 42**  
**Insertion of Anti-Sense Sequences into U1 Operons**



**Figure 43**  
Predicted Secondary structures for U1  
Transcripts with Anti-sense Substitutions



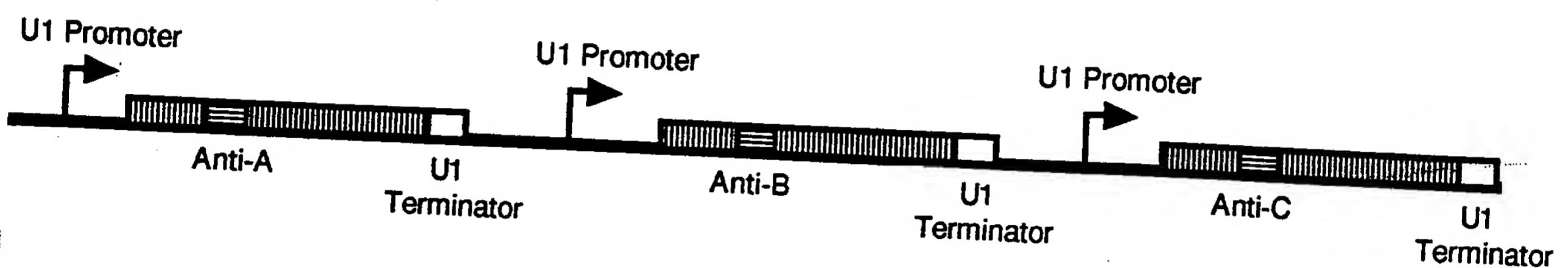
**Figure 44**  
Construction of U1 Multiple Operon Clone



**Figure 45**  
Construction of T7 Triple Operon

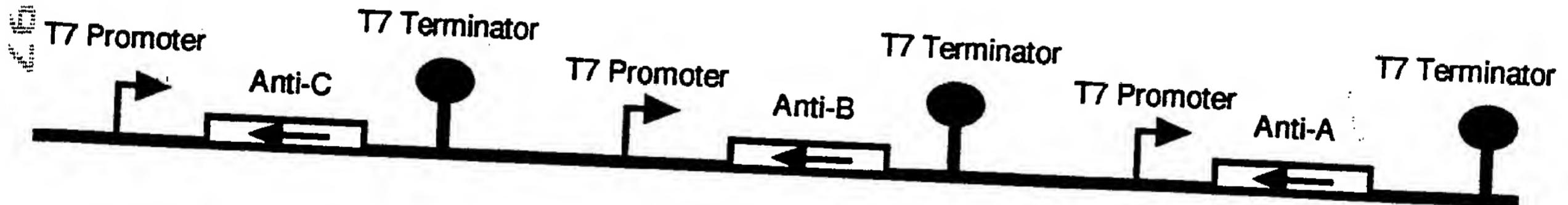
## pNDU1(A,B,C)

Triple U1 Operon Construct with HIV Anti-Sense



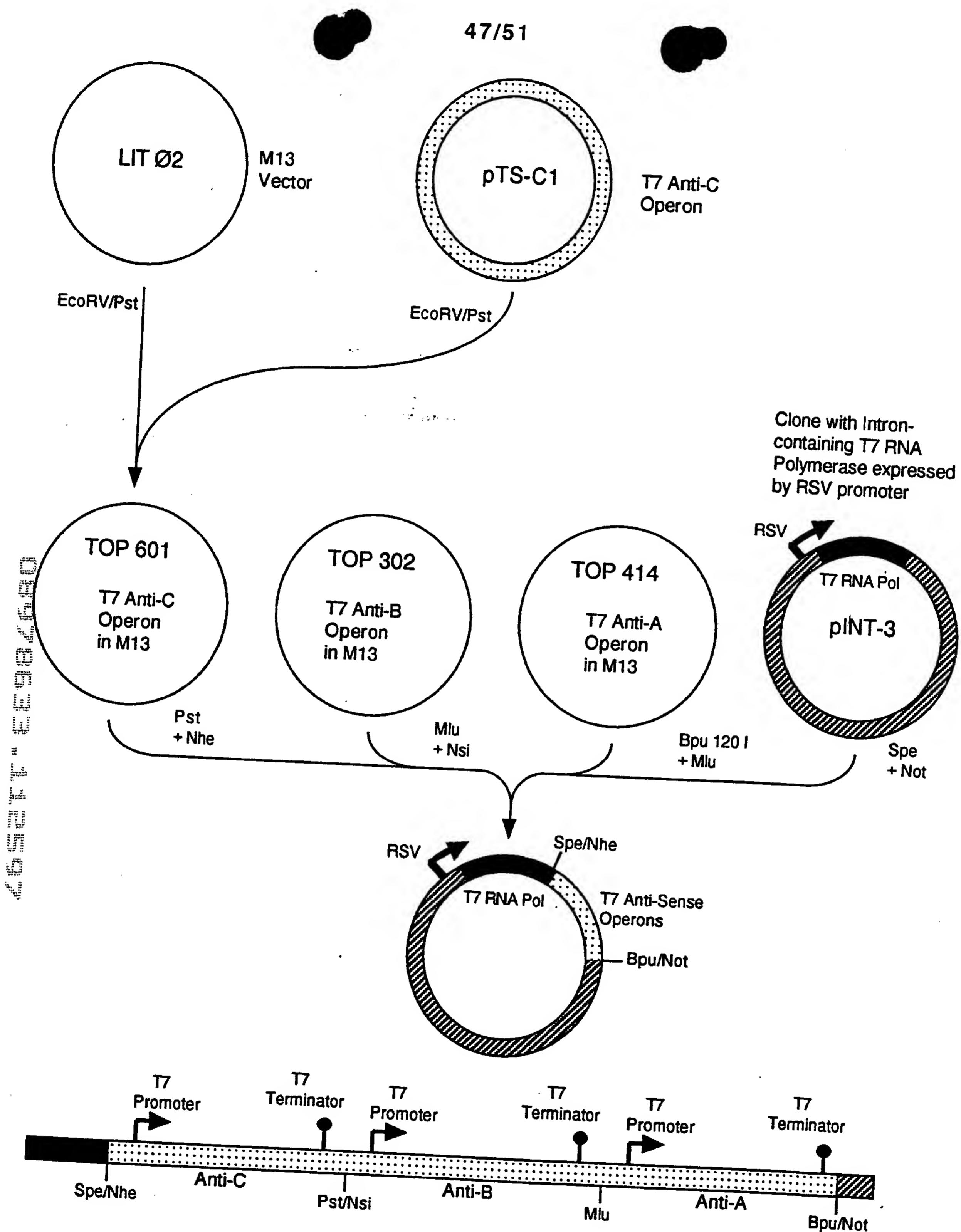
## TRI 101

Triple T7 Operon Construct with HIV Anti-Sense

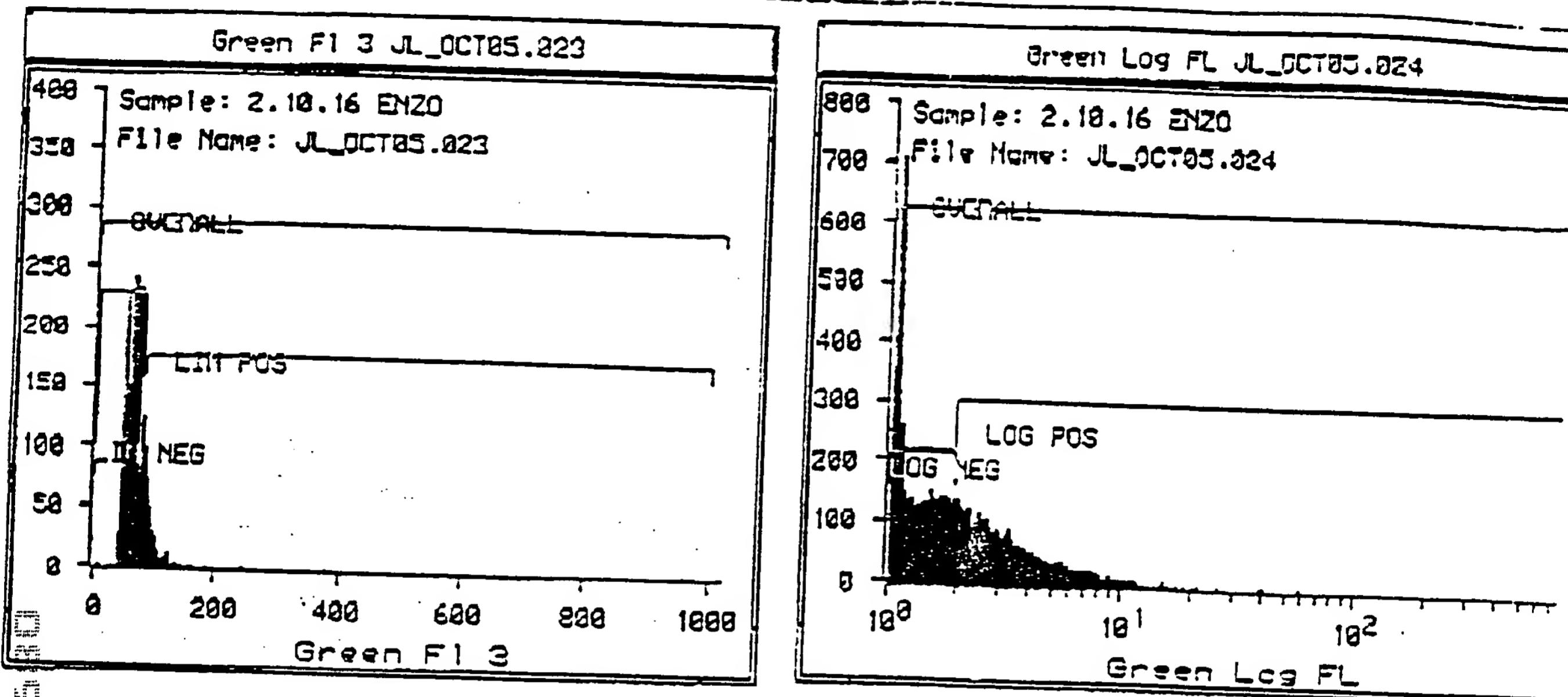


**Figure 46**

Structures of Triple Operon Constructs  
from Figures 44 and 45



**Figure 47**  
Construction of Multiple T7 Operons in Vector coding for T7 RNA Polymerase



Global Statistics									
1. Green F1 3 JL_OCT05.023					Total = 7589				
2. Green Log FL JL_OCT05.024					Total = 7589				
Hist	Region	Bounds	Counts	x	Mean X	Mean Y	Mode	x0	
1.	LIN NEG	1 78	5714	76.1	63.65		78	14	
	LIN POS	85 1002	1129	15.0	97.34		85	17	
	OVERALL	1 1024	7589	100.0	78.28		70	23	
2.	LOG NEG	2 2	4211	56.1	2.34		2	21	
	LOG POS	2 1001	3487	45.4	4.76		3	69	
	OVERALL	2 1001	7589	100.0	3.43		2	88	

Figure 48

Flow cytometry data measuring binding of anti-CD4+ antibody to HIV resistant U037 cells

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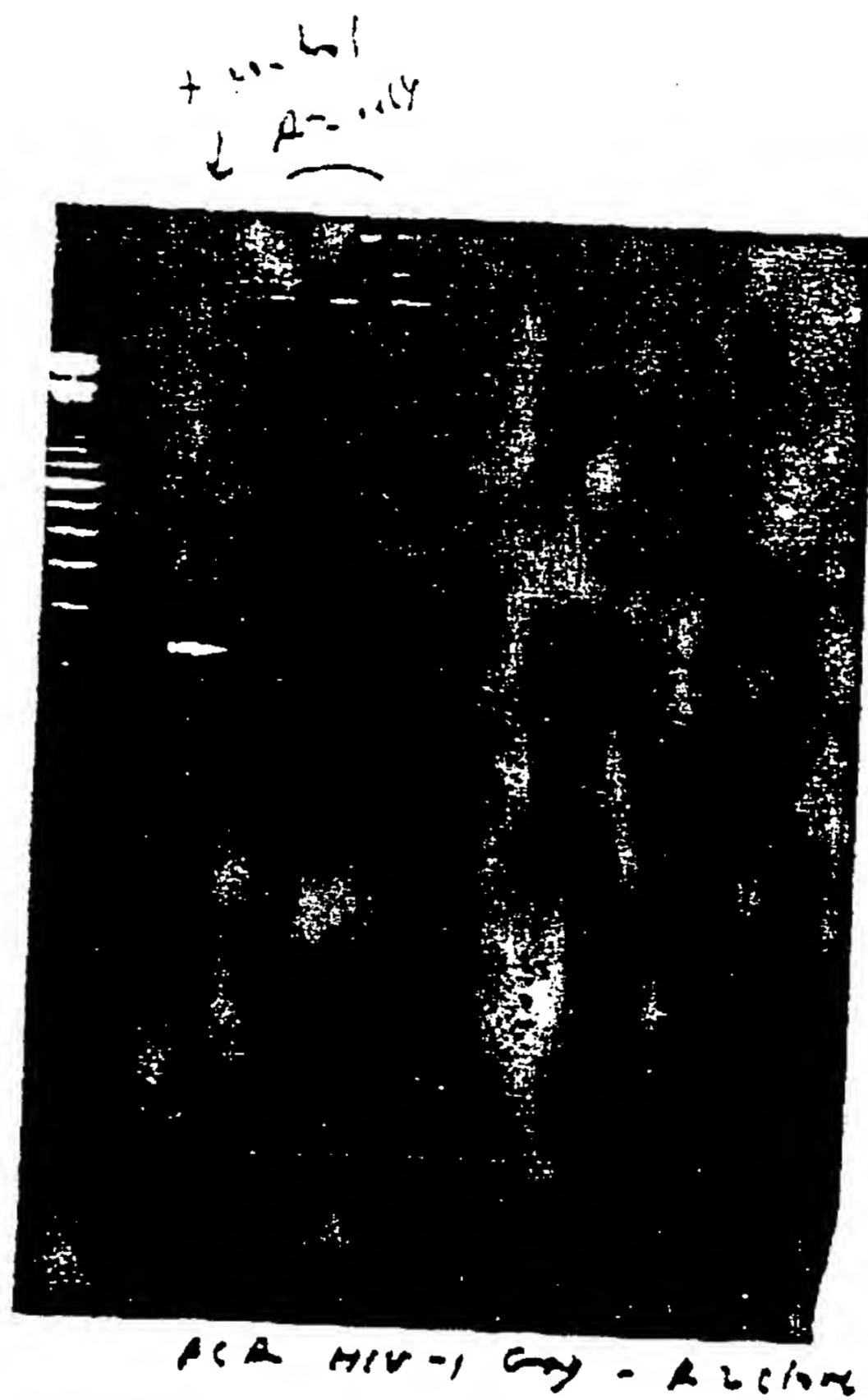
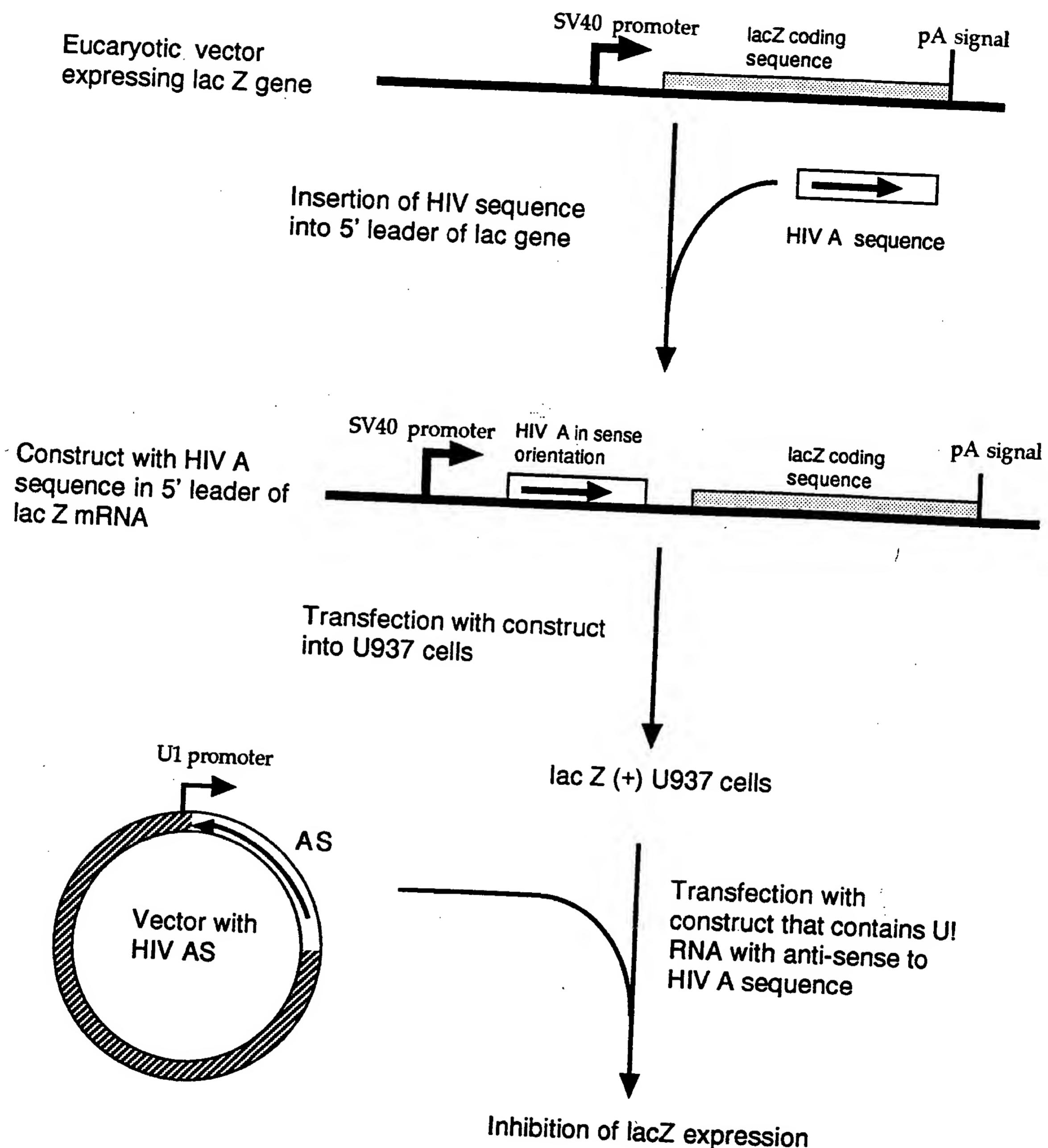


Figure 49

PCR amplification of gag region  
indicating absence of HIV in  
viral resistant cell line (2.10.16)  
after challenge

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**Figure 50**

**Clone with target-lacZ fusion will have reduced expression of lacZ after transfection by HIV Anti-sense construct**

Enzyme activity as expressed by  $A_{420}$  readings  
in extracts prepared from

	$2.5 \times 10^4$ cells	$5 \times 10^4$ cells	$1.0 \times 10^5$ cells
U 937 [untransfected]	0.018	0.023	0.034
U 937 [ HIV A clone ]	0.154	0.277	0.566
U937 [ HIV A / Anti-A ]	0.010	0.017	0.027
U 937 [ HIV A/Anti-ABC ]	0.013	0.021	0.035
U 937 [ HIV A / Null DNA ]	0.120	0.212	0.337

**[ B] Expression of Beta-galactosidase activity by *In situ* assay :**

U 937 [ untransfected ] no blue spots in cells

U 937 [ HIV A clone ] blue spots in cells

U 937 [ HIV A/Anti A ] no blue spots in cells

U 937 [ HIV A/Anti ABC ] no blue spots in cells

U 937 [ HIV A / Null DNA ] blue spots in cells

Figure 51

Expression of Beta-galactosidase activity  
in extracts